LHRH RECEPTOR REGULATION AND DIRECT EFFECTS OF LHRH ON FOLLICULAR STEROIDOGENESIS

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for Frank

the one that got away.

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DECLARATION

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author.

I declare that this was totally composed by myself, and that all the experimental work described herewith was performed by myself except where acknowledgement is made.

No part of this work is being submitted for any other degree and all the studies were carried out at MRC, Reproductive Biology Unit, 37, Chalmers Street, Edinburgh, EH3 9EW.

R.M. Popkin

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ABSTRACT

The studies described in this thesis set out to investigate three areas of LHRH action:- (1) Identification of the factors regulating LHRH receptor concentrations in pituitary and ovary; (2) Investigation of the nature of the short-term effects of LHRH on follicular steroidogenesis; (3) Assessment of the relevance of direct ovarian actions of LHRH in the human.

An LHRH receptor assay capable of quantitative determination of LHRH receptor numbers in individual rat anterior pituitary glands and ovaries was validated and used to investigate receptor changes under a number of reproductive states. In the male, active immunization against testosterone or castration resulted in increased pituitary LHRH receptor concentrations whereas hyperprolactinaemia or removal of endogenous LHRH by active or passive immunization against LHRH was associated with decreased receptors. In the female, ovariectomy or active immunization against LH raised receptors. Active immunization against LHRH decreased receptors. During the oestrous cycle passive immunoneutralization of LHRH, but not LH, prevented the pro-oestrous receptor rise but neither regime prevented the fall to oestrous levels.

It was concluded from these observations that pituitary LHRH receptors are primarily under positive autoregulatory control.

Specific LHRH receptors were characterized and localized by autoradiography in ovarian tissue. Receptor concentrations were increased after active immunization against LH or LHRH and in rats exposed to constant light. Ovarian LHRH receptors were concluded not to be controlled concurrently with those of pituitary.

Basal steroidogenesis was specifically stimulated in a dose dependent manner by LHRH and LHRH agonist in isolated rat pre-ovulatory follicles. Stimulation of progesterone was likely to be mediated by direct granulosa cell action. Basal androgen levels were stimulated, implicating a direct thecal action. However an indirect effect via granulosa cell aromatase could not be excluded.

The question of the existence of an endogenous ovarian LHRH-like material was assessed by measuring specific displacement in radioreceptor and radioimmunoassays by ovarian extracts. Results proved inconclusive due to interference of control tissues and variability of data.

Specific binding of ^{125}I-LHRH agonist was demonstrated to human luteal, but not post-menopausal ovarian tissue. Binding was of low affinity (Ka 3 x 10^7 M^{-1}) and the physiological significance of the observed binding is uncertain.

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CHAPTER 1

GENERAL INTRODUCTION

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1.1 BACKGROUND

The isolation, purification and characterization of luteinizing hormone releasing hormone (LHRH) from bovine and porcine hypothalami (Schally & Bowers, 1964; Matsuo et al., 1971; Schally et al., 1971; Burgus et al., 1972) represented a landmark in reproductive biology. The identity of the stimulatory controlling factor for gonadotrophin release from the anterior pituitary was now known and the theory of hypothalamic control of pituitary function (Harris, 1948) confirmed. LHRH is a decapeptide of molecular weight 1183, its amino acid sequence (pyro-glu-his-trp-ser-tyr-gly-leu-arg-pro-gly-NH_2) is highly conserved throughout evolution (King & Millar, 1980) indicating its vital importance to reproduction.

The essential role of LHRH in the stimulation of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) was elegantly demonstrated by neutralization of LHRH by antibodies. Specific immunoneutralization of LHRH resulted in immediate abolition of the stimulus for pituitary control of gonadal function (Fraser & Gunn, 1973; Koch et al., 1973; see Fraser, 1980 for review). The availability of antisera to LHRH, together with techniques for sampling portal blood, subsequently enabled demonstration of immunoreactive LHRH in the portal blood in rats (Fink & Jamieson, 1974). The physiological importance of endogenously released LHRH in the induction of the LH surge was confirmed by the demonstration that a surge of LHRH occurs coincident with increases in LH in rats, (Sarkar et al., 1976; Levine & Ramirez, 1982) rhesus monkeys, (Neill et al., 1976) rabbits (Tsou et al., 1977) and ewes (Levine et al., 1982).

1.2 REGULATION OF LHRH

Following the identification of LHRH and the development of sensitive immunoassays for its measurement in portal blood (e.g. Nett et al., 1973) major advances were made in the elucidation of its mode of action (for review see Fink, 1979). Clearly two loci were available for control of pituitary function, namely the hypothalamus, as the site of LHRH release, and the pituitary, as the site of action.

1.2.1 LHRH Release

Gonadotrophin release was known to be under central neural control (e.g. see Everett, 1964). This release was elegantly shown to be LHRH-mediated by Fink & Jamieson in 1974, who demonstrated increased release of LHRH into portal blood after electrical stimulation of the pre-optic area. In addition, since the LHRH released in response to stimulation varied during the oestrous cycle (Fink & Jamieson, 1976) it was likely that peripheral factors could influence LHRH release. Oestrogen was subsequently shown to increase responsiveness of neurons releasing LHRH (Sherwood et al., 1976) and to facilitate LHRH release into portal blood (Sarkar & Fink, 1979). * Steroids are capable of modulating the firing rate of hypothalamic neurons (Hayward, 1977; Fink & Geffen, 1978) making it likely that LHRH release is effected by common neuronal pathways. The nature of these pathways is complex and largely a mystery involving dopaminergic, serotonergic and noradrenergic neurones (review by Fink & Geffen, 1978; Barraclough & Wise, 1982).

1.2.2 Pituitary Responsiveness

Numerous studies have shown that pituitary responsiveness to

* Ovariectomy has been shown to result in an increase in portal blood levels of LHRH in the rat thus confirming the importance of steroids in the control of LHRH release (Sherwood and Fink, 1980).

LHRH varies under different endocrine environments (reviewed in Chapter 4). The role of gonadal steroids as both 'negative' and 'positive' feedback regulators of pituitary function is widely acknowledged (Everett, 1964; Knobil, 1974; Aiyer & Fink, 1974; Fink, 1979), the mechanism for these actions being mediated in part by a determination of pituitary responsiveness to LHRH (Aiyer & Fink, 1974; Aiyer et al., 1974a; Fink, 1979 for review).

In addition it was observed that the magnitude of LH released in response to a given dose of LHRH was dependent on the degree of prior exposure to LHRH - a phenomenon termed the 'self priming effect' (Aiyer et al., 1973, 1974b). This was also observed following LHRH release induced by stimulation of the preoptic area (Fink et al., 1976) and was found to be dependent upon protein and mRNA synthesis (Pickering & Fink, 1976).

There are three potential mechanisms by which gonadal steroids and LHRH could influence pituitary sensitivity; the first is by influencing LHRH release (i.e. at a 'pre-receptor' locus), the second by influencing the number and/or affinity of pituitary receptors for LHRH (i.e. receptor regulation) and finally by influencing the storage and release of gonadotrophins (i.e. a 'post-receptor locus'). Chapter 4 of this thesis is concerned with an examination of the second mechanism - namely receptor regulation.

1.3 PARADOXICAL ANTIFERTILITY EFFECTS OF LHRH

With the wealth of data available on the nature and control of gonadotrophin release from the pituitary it was postulated that synthetically produced LHRH would provide a major advance in the induction of fertility. However, toxicity trials during the mid

1970's using synthetic LHRH demonstrated the somewhat surprising findings, that exogenously administered LHRH did not invariably stimulate gonadal function. Administration of pharmacological doses of LHRH (1 mg/day) on d 1-7 of pregnancy inhibited pregnancy in rats (Corbin & Beattie, 1975). In immature male rats, chronic LHRH treatment resulted in decreased seminal vesicle and ventral prostate weight in addition to decreased serum testosterone (Oshima et al., 1975). Consecutive daily administration of LHRH to ewes resulted in diminished LH responses (Rippel et al., 1974) and infusion of LHRH actually inhibited LH release in ovariectomized ewes (Piper et al., 1975). Antifertility effects of LHRH were also noted in rabbits (Rippel & Johnson, 1976a) and hamsters (Windsor et al., 1977).

Initially these observations were ill understood and largely ignored until new LHRH analogues were developed. Although a great deal of effort had been channelled into attempts at synthesizing antagonist analogues, only relatively recently have potent antagonists proved effective at blocking fertility (Rivier et al., 1981; Zarate et al., 1981). The major advances both in clinical uses and in understanding the antifertility effects of LHRH, have come from the development of potent agonist analogues.

In trials designed to test the effectiveness of an antagonist analogue in inhibiting pregnancy in the rat, the LHRH agonist 'control treatment' proved more effective than the antagonist, when administered both pre-(d 1-7) and post-(d 7-12) implantation (Corbin et al., 1978; Bex & Corbin, 1981). The first detailed analysis of antifertility effects of LHRH agonist was the demonstration that

chronic administration to both immature and adult female rats delayed vaginal opening, arrested uterine growth and caused oestrous cycles to cease (Johnson et al., 1976a). These observations have subsequently been confirmed by numerous other studies (Corbin et al., 1978; Rivier et al., 1978; Vilchez-Martinez et al., 1979; Cusan et al., 1979; Maynard & Nicholson, 1979)

Similar inhibitory effects were noted in male rats. Following chronic agonist treatment, testicular weight decreased and spermatogenesis became impaired (Pelletier et al., 1978; Rivier et al., 1979; Cusan et al., 1979) presumably due to decreased testosterone production (Sandow, 1978b; Labrie et al., 1980).

1.4 CLINICAL USES OF LHRH AND ITS ANALOGUES

From the above discussions manipulation of pituitary exposure to LHRH clearly has enormous potential in the regulation of human fertility. The dual uses of LHRH and its analogues either to mimic the endogenous molecule and thus promote fertility, or to utilize the paradoxical inhibitory actions, are amply illustrated by the clinical uses of LHRH.

1.4.1 Restoration of fertility

Pituitary function had been successfully restored in arcuate nucleus lesioned rhesus monkeys with pulses of LHRH every 1-2 h (Knobil, 1980) or in rams in the inactive breeding season by pulses of LHRH (Lincoln & Short, 1980). Pulsatile administration of LHRH was subsequently used to induce ovulation in infertile women with LHRH deficiency due to Kallman's Syndrome (Crowley & McArthur 1980). In addition pulsatile delivery of LHRH every 60-120 minutes using an automatic portable pump has restored fertility in patients

suffering from secondary amenorrhea, and pregnancies have been reported, following treatment (Leyendeker et al., 1980; Yen, 1983). The treatment with LHRH of men suffering from infertility due to impaired gonadotrophin secretion, has resulted in restoration of potential fertility (Hoffman & Crowley, 1982).

1.4.2 Contraceptive effects

Following observations that continuous administration of LHRH agonists causes infertility effects in rodents and sheep (see 1.3), these findings have been extended to the human. This approach offers a new method of contraception, devoid of the side effects of steroids (see Fraser, 1981a and 1982 for reviews). Daily administration of small doses of LHRH agonist had resulted in the cessation of menstrual cycles and abolition of ovulation in women, rhesus mokeys and stumptailed macaques (Nillius et al., 1978; Vickery et al., 1980; Fraser et al., 1980). Other regimes of LHRH administration were also reported to interfere with ovarian function. High doses of LHRH agonist administered during the early follicular phase resulted in the formation of an inadequate corpus luteum (Sheehan et al., 1982), and during the luteal phase induced premature luteolysis (Casper & Yen, 1979; Lemay et al., 1979).

The contraceptive use of LHRH agonist in males has been less encouraging. Although LHRH agonist reversibly inhibited spermatogenesis the decreased testosterone production resulted in impotence and decreased libido (Linde et al., 1981).

The ability of LHRH agonist to suppress gonadal function has important applications in the treatment of hormone-dependent disorders. Thus "chemical castration" using LHRH agonist has been

used to delay the onset of puberty in patients with precocious puberty (Comite et al., 1981) and to reduce gonadal steroid output in steroid dependent tumours of the breast (Klijn & de Jong, 1982) and prostate (Borgmann et al., 1982; Waxman et al., 1983). LHRH agonist has also been used to relieve patients with endometriosis (Meldrum et al., 1982; Werlin & Hodgen, 1983; Shaw et al., 1983).

The development of antagonist analogues to inhibit LHRH action has been less promising (Schally et al., 1980). However, potent antagonists have recently been shown to inhibit ovulation in the rat (Nekola et al., 1982) and in women (Zarate et al., 1981) as well as inhibiting pulsatile LH secretion in castrate male monkeys (Pineda et al., 1983).

Pulsatile administration of LHRH thus has important clinical applications in the induction of fertility whilst LHRH agonists have a wider clinical potential in suppressing pituitary function.

1.5 MECHANISM OF ANTIFERTILITY EFFECTS OF LHRH

Whereas the induction of fertility by LHRH is well understood, (its rationale being based on the physiology of the endogenous peptide) the mechanisms by which LHRH administration results in inhibition of fertility are largely unknown. However, at least three explanations have been suggested, namely pituitary desensitization, gonadal desensitization and direct gonadal actions.

1.5.1 Pituitary desensitization

Continuous or repeated stimulation of the pituitary with LHRH eventually results in a decreased pituitary responsiveness to LHRH (Belchez et al., 1978; Sandow, 1978a; Sharpe et al., 1979; Fraser et al., 1980). This phenomenon of desensitization has been observed for

a number of peptide hormones (see Catt 1979 for review) and is generally considered to be due to a decrease in the number of receptors for that ligand i.e. down-regulation (Raff, 1976; Catt, 1979). It is therefore possible that continuous exposure of the gonadotroph cells to LHRH or LHRH agonist causes down-regulation of its receptors and subsequent desensitization, with decreased gonadotrophin release and impairment of gonadotrophin dependent functions, such as spermatogensis and ovulation. This possibility is examined in Chapter 4.

1.5.2 Gonadal desensitization

Since chronic treatment with LHRH agonist initially results in stimulation of supraphysiological levels of LH (e.g. Fraser & Lincoln, 1980) it has been suggested that gonadal steroidogenic impairment could be induced by continuously high circulating gonadotrophin levels following agonist treatment (Auclair et al., 1977a,b; Cusan et al., 1979). High LH levels are known to result in desensitization of the gonad due to receptor down-regulation in males and females (Sharpe, 1976; Conti et al., 1976). Chronic administration of LHRH agonist was associated with 70% inhibition of testicular LH receptors (Auclair et al., 1977b; Cusan et al., 1979) and the post coital antifertility effects of LHRH agonist were associated with decreased ovarian LH receptors (Kledzik et al., 1978).

However, if this mechanism were of primary importance in determining the antifertility effects of LHRH it would be expected that exogenous administration of gonadotrophins would induce similar antifertility effects to those of LHRH agonist. Abnormally high gonadotrophin levels have been demonstrated to disrupt both

follicular development, ovulation (Friedrick et al., 1975) and testicular function (Hsueh et al., 1977) but whereas the effects of chronic hCG administration are transient, those of LHRH are prolonged (Cusan et al., 1979). In addition the LHRH - induced decrease in progesterone production in pregnant rats could not be repeated with high LH administration (Yoshinaga, 1979). Moreover, whilst daily administration of LHRH agonist resulted in inhibition of testosterone production and decreased secondary sex organ weights, daily PMSG treatment increased both parameters (Rivier et al., 1979). These data suggest therefore that the antifertility effects of LHRH cannot be due entirely to the secondary induction of supraphysiological levels of LH and subsequent gonadal desensitization.

1.5.3 Direct gonadal actions of LHRH

The above explanations for the mechanism of the paradoxical antifertility effects of LHRH are based on the premise that LHRH acts exclusively on the pituitary gland. This assumption was challenged in 1976 when Rippel & Johnson (1976b) showed that the hCG-induced ovarian and uterine weight gain in hypophysectomized rats could be inhibited by concurrent administration of LHRH agonist, thus implicating a direct ovarian site of action for LHRH agonist. This important observation went largely unnoticed and it was three years before direct gonadal effects were re-examined. The findings of Rippel & Johnson were subsequently confirmed and extended by Ying & Guillemin (1979a) who in 1979 showed that follicular maturation and ovulation induced by PMSG in hypophysectomized rats could be prevented by LHRH agonist in a dose-dependent manner. Exciting confirmation of direct ovarian actions of LHRH come from in vitro

experiments showing LHRH inhibition of FSH-induced oestradiol production in isolated granulosa cells (Hsueh & Erickson 1979a). This effect was specific for LHRH, being prevented by LHRH antagonists (Hsueh & Ling, 1979). An important contribution to this data was the observation that the actions of LHRH were associated with specific competative uptake of labelled LHRH agonist by the ovary, thus suggesting the presence of specific binding sites (Mayar et al., 1979). Evidence for the existence of such receptors as well as a discussion of their regulation is presented in Chapters 3 and 5.

Direct gonadal actions of LHRH were not however confined to the ovary. LHRH was shown to inhibit FSH-induced increases in testicular weight and steroidogensis in hypophysectomized male rats (Hsueh & Erickson, 1979b).

Few studies have attempted to elucidate the relative importance of pituitary and extrapituitary sites of action in determining the antifertility effects of LHRH. Since LHRH agonist administration to hypophysectomized rats results in an inhibition of gonadal LH/hCG receptors (Arimura et al., 1979; Bambino et al., 1980), gonadal desensitization following LHRH administration to intact rats could be due to either direct or pituitary mediated effects. A comparison of the gonadal LH receptors 48 hrs after a single injection of LHRH agonist in intact versus hypophysectomized rats (Séguin et al., 1981) provided interesting data on this question. All treated animals showed decreased receptors relative to controls. The receptor decrease was greater in intact than hypophysectomized male rats, but both intact and hypophysectomized female rats showed similar

responses. The authors concluded that the major cause of testicular LH receptor loss was due to down-regulation following hypersecretion of LH. However in the female, direct gonadal actions were likely to play a more important role (Séguin et al., 1981).

In conclusion, the antifertility effects observed after exogenous administration of LHRH and its analogues are likely to be due to complex multiple interactions involving pituitary and gonadal desensitization together with direct gonadal actions. The relative contribution of each, particularly in the case of the human, remains uncertain.

1.6 EXTRAPITUITARY ACTIONS OF LHRH

One of the most intriguing observations to emerge from studies outlined above was that LHRH and its agonists were capable of directly regulating tissues other than the pituitary. Since further studies on this theme were a major part of this thesis the following sections consider the evidence for extrapituitary actions.

1.6.1 Direct Ovarian Actions

1.6.1.1 Granulosa Cells

In vitro studies on the action of LHRH on isolated granulosa cells have emphasised an inhibitory effect. Thus, LHRH and LHRH agonist inhibited FSH-induced increases in oestradiol and progesterone production (Hsueh & Erickson, 1979a; Knecht & Catt, 1981; Ranta et al., 1982). This inhibition was manifested by doses of LHRH compatible with the Ka of the ovarian LHRH receptor (see Chapter 3). Similar inhibition has been observed in hypophysectomized rats treated with FSH and LHRH (e.g. Jones and Hsueh, 1981a). These data are not confined to the rat; direct inhibition of

granulosa cell function has been reported in porcine granulosa cells treated with LHRH agonist (Massicotte et al., 1980).

Since FSH-induction of steroidogenesis involved changes in the activities of a number of steroidogenic enzymes it seemed likely that LHRH would reverse these changes. This has been demonstrated to be the case for four such enzymes.

Progesterone release is primarily under the control of three enzymes: (a) 3β hydroxysteroid dehydrogenase (3βHSD) catalysing the synthesis of progesterone from its immediate precursor, pregnenolone; (b) 20α hydroxysteroid dehydrogenase (20α HSD) responsible for the conversion of active progesterone to its inactive 20 hydroxypregn-4-en-3-one (20α DHP) metabolite; and (c) sidechain cleavage (SCC) enzyme which determines the availability of pregnenolone from its precursor cholesterol. LHRH was found to inhibit FSH-induced increases in 3βHSD (Hsueh & Jones, 1982a; Jones & Hsueh, 1982) and to stimulate 20α DHP (Jones & Hsueh, 1981b,c) activity. In addition, since FSH-induced synthesis of pregnenolone from cholesterol was inhibited by LHRH (Hsueh & Jones, 1981; Jones & Hsueh, 1982) an effect on SCC enzyme seems likely. Thus LHRH antagonised all three parameters of FSH-induced changes in progesterone metabolism, resulting in decreased rate of synthesis and increased rate of inactivation.

Oestradiol production is controlled primarily by the availability of androgen precursor and on the activity of aromatase. Again LHRH and LHRH agonist have been found to inhibit FSH-induced increases in aromatase activity (Hsueh et al., 1980; Hiller et al., 1981; Gore-Langton et al., 1981). In addition to changes in the V

max of FSH-dependent enzymes (Hsueh et al., 1980; Jones & Hsueh, 1981), LHRH inhibited the FSH-induced increase in both LH (Hsueh et al., 1980) and prolactin (PRL) receptors (Navickis et al., 1982).

LHRH and LHRH agonist were also shown to influence granulosa cell morphology. Both FSH-induced cellular aggregation and microvillus formation were inhibited by LHRH and LHRH agonist (Amsterdam et al., 1981; Knecht et al., 1982). Since high resolution autoradiographic studies have suggested that new receptors are associated with microvillus formation (Amsterdam et al., 1981) it is likely that LHRH-induced receptor decreases are mediated by such morphological changes. Interestingly, LHRH inhibits FSH-induced increases in membrane fluidity (Strulovici et al., 1981), again confirming an inhibitory influence on the dynamics of membrane function.

In conclusion the overall picture to emerge from analysis of the effects of LHRH and LHRH agonists on granulosa cell function was an inhibitory one, confirming the antifertility effects discussed earlier. Some of these inhibitory actions are summarizied in Fig. 1.1 and clearly involve complex effects on both steroidogenic and morphological responses. However, the majority of studies discussed have utilized prolonged exposure of isolated cells to LHRH or its agonist (see Hsueh & Jones, 1981 for review) on gonadotrophin mediated events. If LHRH plays a physiological role within the ovary (as suggested in 1.6.7) the short-term effects may be equally, or perhaps more, important. This hypothesis provided the basis for experiments detailed in Chapter 7.

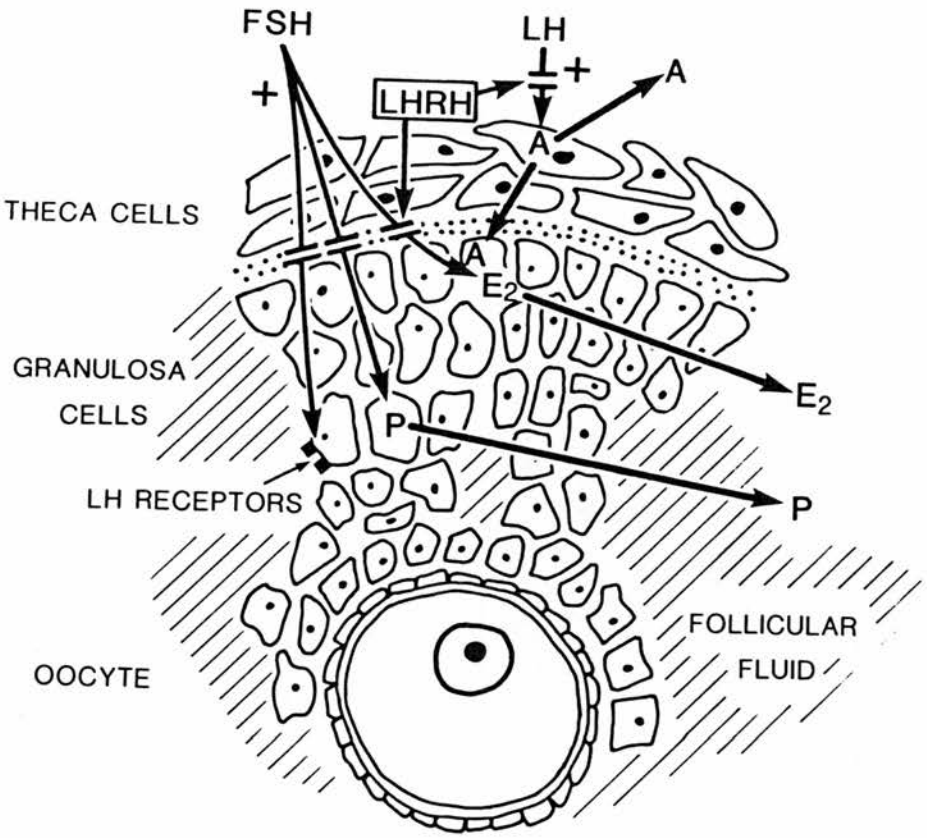

FIGURE 1.1 Summary of some of the inhibitory actions of LHRH on ovarian follicular function, involving inhibition of FSH induced increases in oestradiol (E_2), progesterone (P) and LH receptor formation and in LH induced increases in androgen (A) production.

1.6.1.2 Luteal cells

Two in vitro models have been utilized to provide a source of luteal tissue. The first involved induction of "granulosa-luteal" cells with functional LH receptors, following prolonged exposure of granulosa cells to FSH (Erickson et al., 1979). LH-induced steroidogenesis and prolactin receptor formation were inhibited by LHRH both in vitro and in vivo (Jones & Hsueh, 1980). The second model utilized luteal cells formed in vivo after treatment of immature rats with PMSG/hCG. This model confirmed the inhibitory effect of LHRH and LHRH agonist on LH-induced progesterone production (Clayton et al., 1979a; Harwood et al., 1980a; Behrman et al., 1980; Massicotte et al., 1981; Hall & Behrman, 1981).

Interestingly, LHRH agonist administration to hypophysectomized PMSG/hCG primed rats resulted in decreased LH receptors (Harwood et al., 1980b; Behrman et al., 1980; Hall & Behrman, 1981). Thus the LHRH- and LHRH agonist-induced reduction in luteal cell sensitivity to LH could be mediated by decreasing LH receptor numbers.

An important insight into the mechanism by which such direct luteal effects could be mediated came with the demonstration of specific high affinity LHRH receptors on luteal cells (Clayton et al., 1979; Harwood et al., 1980a)

1.6.1.3 "Interstitial"/"Prepubertal tissue"

Preincubation of collagenase-dispersed cells from 25 day old rat ovaries with LHRH agonist decreased the progesterone response induced by hCG, cholera toxin or 8 bromo cAMP in short term culture in vitro (Reddy et al., 1980). Similar cells from hypophysectomized, 21 day old rat ovaries incubated in prolonged cultures indicated that LHRH

inhibited the LH-, cholera toxin-, dibutyryl cAMP or oestrogen - induced increase in "aromatizable androgen production" (Magoffin et al., 1981). In addition LHRH agonist inhibited steroidogenesis in hamster interstitial cells (Silavin & Greenwald, 1982). Thus LHRH appears to have the potential to influence directly the steroidogenic output of a number of cell types within the rat ovary.

1.6.2. Direct testicular actions of LHRH

Confirmation of direct testicular effects of LHRH following in vivo observations in hypophysectomized rats (Hsueh & Erickson, 1979b; Arimura et al., 1979) came with the demonstration of specific high affinity LHRH receptors in isolated rat Leydig cells (Sharpe & Fraser, 1980a) and testicular homogenates (Bourne et al., 1980; Perrin et al., 1980a). Since these receptors were confined exclusively to Leydig cells, the direct effects of LHRH and LHRH agonists on testicular steroidogenesis were mediated entirely through Leydig cells. These actions were inhibitory, decreasing the sensitivity of Leydig cells to FSH (Bambino et al., 1980; Hsueh et al., 1981).

Little is known about the effects of LHRH on steroidogenic enzymes within the testis although a decrease in 17α hydroxylase activity has been reported (Bambino et al., 1980). Since LHRH receptors are absent from Sertoli cells, the lack of effect of LHRH on FSH- cholera toxin- and dibutyryl cAMP - stimulation of aromatase activity is therefore not surprising (Gore-Langton et al., 1981; 1982).

Direct testicular actions of LHRH confirmed the antifertility effects of LHRH observed in male rats (1.3): In contrast to the ovary

however, LHRH had only one site of action within the testis, namely the Leydig cell.

1.6.3 Direct Uterine Action

A direct uterine action has been postulated for LHRH (Jones, 1979; Sundaram et al., 1981). However, since no evidence has been found for specific uterine LHRH receptors (e.g. Clayton & Catt, 1981b) the physiological significance of this hypothesis remains questionable.

1.6.4. Direct effects on the Placenta

Preliminary evidence suggested that LHRH was present in the placenta and that LHRH stimulated placental hCG production (Khodr & Siler-Khodr, 1978a; Siler-Khodr & Khodr, 1981) and released hCG from trophoblast cells (Bützow, 1982). However, this result has not been confirmed by other investigators (e.g. Hussa, 1980) although LHRH inhibition of progesterone (Wilson & Jawad, 1980) and oestrogen (Branchaud et al., 1983) have been reported. Specific placental binding sites have been demonstrated in human placental tissue (Currie et al., 1981), but these are of low affinity (Ka 5 x 10^7 M^{-1}) and render it unlikely that concentrations of LHRH sufficiently high to activate such receptors, would occur in vivo. Thus the role of LHRH in the control of placental function has yet to be adequately elucidated. However, it is of interest that the LHRH decapeptide has now been characterized from human placenta extracts (Tan & Rousseau, 1983).

1.6.5 Direct effects on Tumours

The ability of LHRH to inhibit steroid-dependent tumours (De sombre et al., 1976; Johnson et al., 1976b) was explained

initially on the basis of its "chemical castration" effects and reduction of gonadal steroidogenesis. However, this may not be the sole explanation since there are reports of direct effects of LHRH on chorionic gonadotrophin release from rat choriocarcinoma cells and human mammary tumour cells (Teodorczyk-Injeyan et al., 1981). In addition, LHRH agonist inhibited testosterone release from a Sertoli-Leydig cell tumour (Lamberts et al., 1982).

1.6.6 LHRH and the Nervous System

LHRH may function as a peptide neurotransmitter influencing the function of neurons outside the hypothalamic area at least in the rat (Moss, 1979). For example LHRH has been detected in extrahypothalamic areas (see Samson et al., 1980). Moreover, centrally-mediated effects of LHRH on sexual behaviour have been reported (e.g. Pfaff, 1973; Moss & McCann, 1973; Sakuma & Pfaff, 1980 and see Mauk et al., 1980 for review). An antagonist analogue of LHRH has also been shown to suppress sexual behaviour in rats (Dudley et al., 1980).

In addition to the central nervous system, LHRH has been detected in sympathetic neurons in the frog (Jan et al., 1979). However, the presence and role of LHRH in mammalian sympathetic neural control remains to be demonstrated.

1.6.7 Physiological Significance

Since hypothalamic LHRH is rapidly degraded (e.g. Jeffcoate et al., 1974) it is unlikely that circulating levels sufficient to interact with gonadal LHRH receptors, and therefore to exert effects on gonadal function, are attained. The presence of specific actions, mediated by high affinity receptors, particularly on gonadal

cells (see Sharpe, 1982 for review) has led to the suggestion that, far from being a pharmacological manifestation due to the administration of high doses of LHRH or LHRH agonist, the direct gonadal effects are of physiological relevance. Thus the gonads have been suggested to produce an endogenous LHRH or LHRH-like molecule, which under normal circumstances would act locally and represents the true ligand for the receptors (Ying & Guillemin, 1979a).

There is ample evidence for the presence of non-steroidal regulators of granulosa cell function (see Channing, 1979 for review). Some of the effects reported for such factors bear a considerable resemblance to the characteristic inhibition of gonadotrophin-induced changes by LHRH. For example, LHRH inhibited the FSH-induced increase in cAMP in isolated porcine granulosa cells (Massicotte et al., 1980) and a factor present in porcine follicular fluid also depressed LH-induced cAMP (Ledwitz-Rigby et al., 1977). In addition LHRH decreased basal progesterone production in porcine granulosa cells (Massicotte et al., 1980) and fluid from small porcine follicles has been shown to be inhibitory to basal progesterone (Ledwitz-Rigby et al., 1977 and see Ledwitz-Rigby & Rigby, 1981 for review).

It is therefore possible that a gonadal LHRH-like peptide is present in follicular fluid, regulating follicular function, a hypothesis which is examined further in Chapter 8. This concept is particularly important when considering the wide clinical uses of LHRH (see 1.4). It is not known whether LHRH exerts direct gonadal actions in the human and, if so, whether the dose regimes used for contraception (1.4.2) are exerting inhibitory effects directly on

luteal function. Chapter 6 examines this possibility.

1.7 AIMS OF THESIS

Three main areas of research were seen as being of particular importance in further developing an understanding of the actions of LHRH, at the onset of work described in this thesis, namely: (a) identification of the factors regulating LHRH receptors; (b) investigation of the nature of the short-term direct actions of LHRH on follicular steroidogenesis; and (c) assessment of the relevance of direct gonadal actions of LHRH in the human. Accordingly attention was focussed on the following objectives:-

1) Development of an LHRH receptor assay capable of quantitative measurement of receptors in individual anterior pituitary glands and ovaries (Chapter 3).

2) Utilization of such an assay to examine the regulation of LHRH receptors, particularly in assessing the role of heterologous ligand regulation and autoregulation in the determination of receptors numbers (Chapters 4 and 5).

3) Assessment of whether direct ovarian actions of LHRH occur in the human by investigation of the ability of ^{125}I-LHRH agonist to bind to human ovarian tissue (Chapter 6).

4) Determination of the short-term effects of LHRH and LHRH agonist on follicular function and in particular, the assessment of whether LHRH is capable of influencing thecal function (Chapter 7).

5) Extraction of ovarian LHRH-like material and its characterization using radioreceptor and radioimmunoassay techniques (Chapter 8).

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 RADIOLABELLING OF LHRH
  2.1.1 Introduction
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2.2 IODINATION OF LH

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2.1 RADIOLABELLING OF LHRH

2.1.1. Introduction

Radioreceptor and radioimmunoassays are critically dependent on the availability of a tracer with a high specific activity. The advantages of tritiated ligands are that the structural integrity of the molecule is maintained and that the labelled hormone is stable for storage. Although tritiated LHRH (^{3}H-Pro^{9}LHRH) had been synthesised (e.g. Grant et al., 1973; Perrin et al., 1980b) it was found to produce relatively low yield and low specific activity.

The vast majority of studies now utilize LHRH labelled with one ^{125}Iodine atom at the Tyr position. This preparation has the advantage of high yield and specific activity. However, the incorporation of a large iodine atom necessarily increases the net charge and electrophoretic mobility which may decrease the biological potency of the labelled ligand (Nieman & Sandow, 1973; Terada et al., 1980). Thus, although ^{125}I-LHRH agonist is biologically active in vivo (Sandow, 1978a; 1978b) the activity of the iodinated hormone was estimated at 50% of that of the unlabelled hormone (Sandow, 1978a). This figure is in agreement with the estimated maximum receptor binding ability of the iodinated tracer (Clayton et al., 1980a; Marshall et al., 1980).

Two methods of iodination have been used for LHRH. The first is based on that of Greenwood et al., 1963 with Chloramine T as an oxidizing agent. However, although this method results in the production of monoiodinated LHRH, the specificity is variable (see Table 2.1). The second method was therefore adopted as the procedure of choice for this thesis. This involved a modification of the

TABLE 2.1

Comparison of Specific Activity of Radiolabelled LHRH and LHRH analogues obtained by different methods.

Radiolabelled Compound	Method of Iodination	Specific activity	Reference
^{125}I-LHRH	Lactoperoxidase	1250-1450 µCi/ug	Marshall et al., 1975
^{125}I-LHRH	"	1574 Ci/mMol	Wagner et al., 1979
^{125}I-LHRH agonist	"	850-1500 µCi/µg	Clayton et al., 1979a
^{125}I-LHRH agonist	"	1500 µCi/µg	Hazum et al., 1981b
^{125}I-LHRH agonist	"	1330 µCi/µg	Adams & Spies, 1981a
^{125}I-LHRH agonist	"	800-1000 µCi/µg	Asch et al., 1981
^{125}I-LHRH agonist	"	800-1200 µCi/µg	Pieper et al., 1981
^{125}I-LHRH antagonist	"	1000 µCi/µg	Meidan & Koch, 1981
^{125}I-LHRH agonist	Chloramine T	350 µCi/µg	Giguere et al., 1981
^{125}I-LHRH agonist	"	280- 380 µCi/µg	Marchetti et al., 1982
^{125}I-LHRH agonist	"	850-1250 µCi/µg	Marian et al., 1981
^{125}I-LHRH agonist	"	280- 385 µCi/µg	Reeves et al., 1982
^{125}I-LHRH agonist	"	800 µCi/µg	Smith-White & Ojeda, 1981
^{125}I-LHRH agonist	"	250 µCi/µg	Jones et al., 1980
^{3}H-LHRH		16- 25 Ci/mMol	Perrin et al., 1980a
^{3}H-LHRH		36.5 C/mMol	Grant et al., 1973

lactoperoxidase method utilizing a hydrogen peroxide generating system as described by Miyachi et al., 1973 and had been shown to yield monoiodinated LHRH of high specific activity (Sharpe & Fraser, 1980) (Table 2.1) which is stable for up to 14 weeks (Marshall & Odell, 1975). A glucose/glucose oxidase system provided a continuous source of hydrogen peroxide thus avoiding high denaturing concentration of strong oxidising agents (Miyachi et al., 1973).

2.1.2 Iodination of LHRH agonist

Reagents: Bovine serum albumin (BSA) (Sigma Type A6003 Fraction V)
Glucose (BDH Chemicals Ltd., Poole, England)
Glucose oxidase (Miles Labs Inc., U.S.A.,)
Lactoperoxidase (Sigma, Product No. L8503) 1 μg/10 μl
Sephadex G25 fine (Pharmacia)

Phosphate buffer 0.05 M pH 7.4
Acetic acid buffer 0.01N
Elution buffer 0.01N acetic acid, 0.1% BSA.

$Na^{125}I$ (Amersham) as carrier free solution in NaOH, specific activity 100 mCi/ml

LHRH or LHRH agonist (D-Ser-tertiarybutyl^{6}-des-Gly NH^{10}-LHRH-nonapeptide ethylamide) (Hoechst, Germany) 1mg/ml

Iodination procedure

To a reaction vessel (63 x 11mm polystyrene tube) was added in sequence:-

1. 5 µl LHRH agonist
2. 40 µl 0.05M phosphate buffer
3. 5 µl lactoperoxidase
4. 5 µl glucose oxidase
5. 20 µl $Na^{125}I$
6. The reaction was started by adding 20 µl of 0.1% glucose solution (to activate the glucose oxidase and provide a source of hydrogen peroxide as oxidizing agent).

   The reaction tube was stoppered, vortexed, and left to react for 4 minutes.

7. The reaction was terminated by the addition of 300 µl of elution buffer and the mixture immediately applied to a column of Sephadex gel.

Chromatography

Purification of monoiodinated LHRH agonist was achieved by column adsorption chromatography using Sephadex gel. 12 g of Sephadex G25 fine were left for 12-24 h to swell in the presence of an excess (75 mls) of 0.01 N acetic acid. The swollen gel was carefully poured into a 60 cm x 1 cm glass column and left to settle to a packed height of 45 cms. The column was coated with 10 mls 0.01N acetic acid 5% BSA, to minimise adsorption of iodinated LHRH, and washed with 50 mls 0.01N acetic acid, 0.1% BSA elution buffer.

The contents of the reaction tube were applied to the top of the

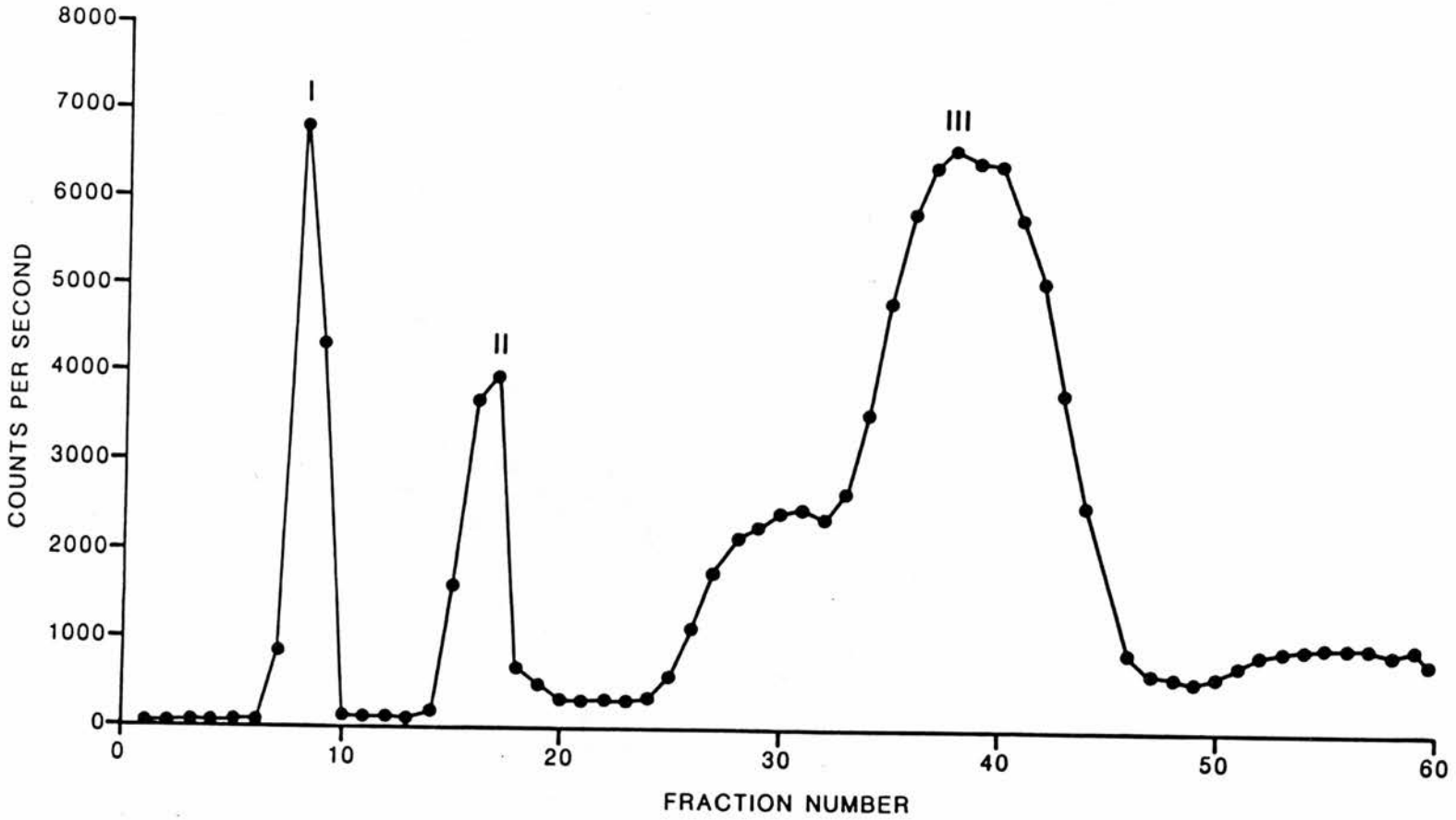

FIGURE 2.1 Elution profile for ^{125}I-LHRH agonist from Sephadex G25 column. Peak III was taken as monoiodinated LHRH agonist. Each point represents a single determination of radioactivity (counts per second).

column using a long Pasteur pipette and allowed to soak into the gel. The reaction vessel was washed with 300 µl of buffer and this was applied to the column as described. The column was fitted with a glass reservoir containing elution buffer. Fractions (70 drops) were collected into glass tubes which had been precoated with 100 µl 0.01 N acetic acid, 5% BSA.

Results

A typical elution profile for iodinated LHRH agonist is shown in Fig. 2.1. The same pattern is seen also for ^{125}I-LHRH. It has been shown that the first peak of radioactivity represents unreacted iodine and subsequent peaks contain iodinated peptides with varying degrees of immunoreactivity, as assessed by the ability of label to bind excess LHRH antibody, with the main peak binding 90% of antibody (Marshall & Odell, 1975; Copeland et al., 1979). The receptor binding ability for each fraction, as assessed in the Leydig cell receptor assay, indicated that maximal binding was associated with the third major peak which was taken to represent the mono-^{125}I-LHRH agonist fraction (Sharpe & Fraser, 1980). The peak fraction together with the following two fractions from the third major peak were pooled and stored in 250 µl aliquots at -20°C. In general the tracer was found to retain binding characteristics for long periods provided a given aliquot was not thawed and refrozen. In practice the tracer was used up to eight weeks after storage with no detectable deterioration in binding ability.

The specific activity of the tracer was assessed by self displacement in either rat Leydig or rat pituitary radioreceptor assay (Fig. 2.2). For the calculation it was assumed that at 50%

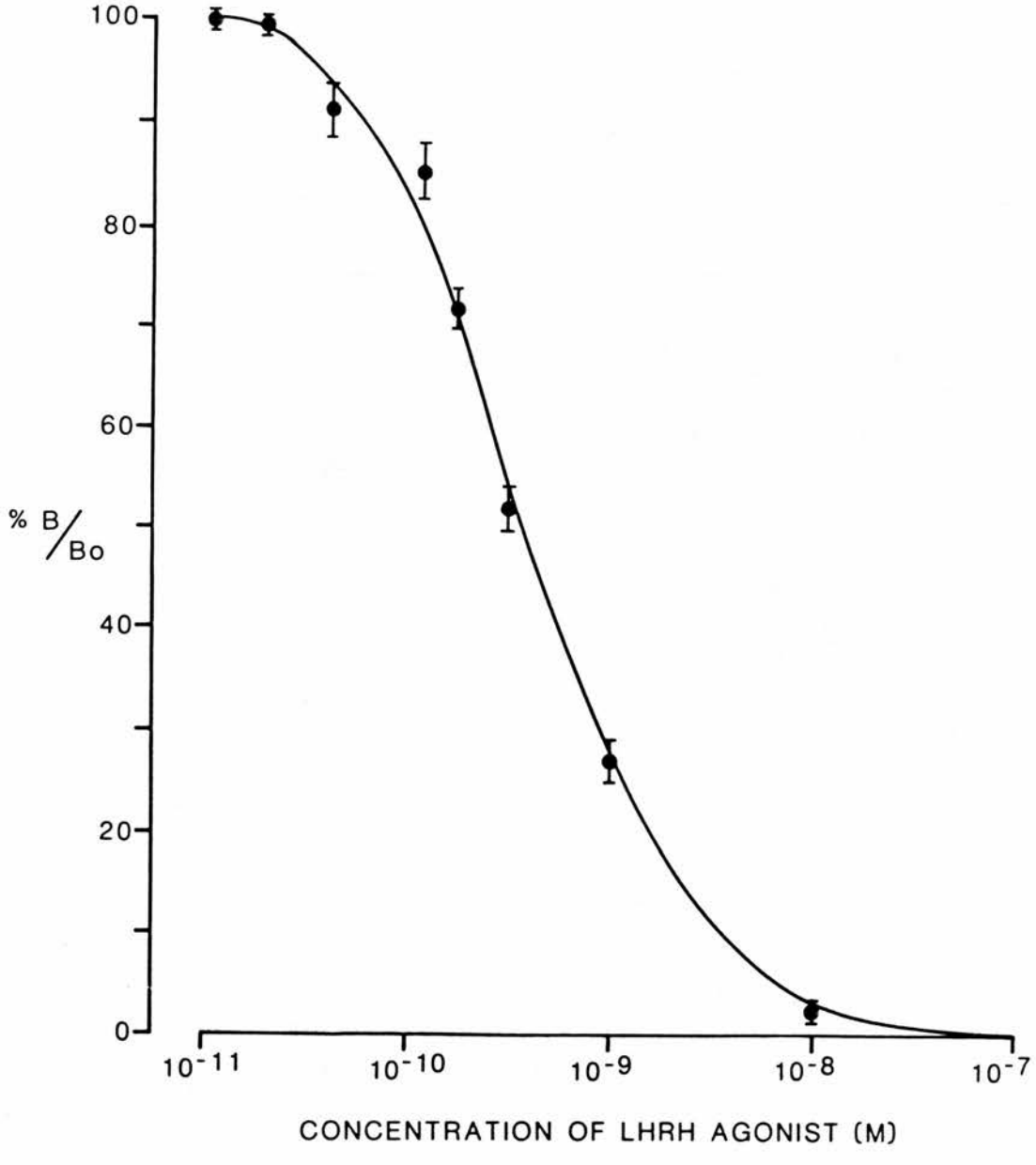

FIGURE 2.2 Displacement of ^{125}I-LHRH agonist binding to rat pituitary homogenate by unlabelled LHRH agonist. Each point represents mean ± range of duplicate determinations.

The specific activity of the tracer was calculated by assuming that at 50% displacement of binding the concentration of cold hormone (3 x 10^{-10}M) was in equilibrium with and thus equivalent to the amount of labelled hormone added (100,000cpm). From this premis the number of counts of labelled hormone equivalent to 1mg cold hormone was calculated and expressed as mCi/mg.

displacement (3 x 10^{-10} M) the labelled hormone (100,000 cpm) is in equilibrium with the unlabelled hormone. The specific activity has been found to range from 900-1200 mCi/mg, figures within the range reported by others utilizing the lactoperoxidase technique (Table 2.1). The theoretical specific activity of monoiodinated LHRH has been calculated to be 1600 µg/ml (Marshall & Odell, 1975) and the range of values obtained using lactoperoxidase are thus consistent with a monoiodinated ligand.

2.2 IODINATION OF RAT LH

Reagents: BSA

$Na^{125}I$

Sephadex G50 (Pharmacia)

Elution buffer 0.01M phosphate buffered saline (PBS)

Chloramine T

Rat LH I-4 NIAMDD, NIH, U.S.A.,

Sodium metabisulphite ($Na_2S_2O_5$) 0.16 mg/ml in 0.05 M PBS

Potassium iodide (KI) 20 mg/ml in 0.05 M PBS

Iodination Procedure

To a reaction vessel (63 x 11 mm polystyrene tube) was added in sequence:-

1. 2 µg LH
2. 20 µl 0.05M PBS
3. 200-350 µCi $Na^{125}I$

TABLE 2.2

Elution Profile of ^{125}I-LH

Fraction No.	Counts/sec.
1	100
2	310
3	340
4	113
5	2436
6	119006
7	4082
8	4190
9	4200
10	2890
11	4860
12	4658
13	213050
14	116716
15	123800
16	4960
17	5030
18	3900

4. The reaction was started by adding 10 µl Chloramine T (5 mg/ml in 0.05 M PBS). React for 20 seconds.
5. The reaction was terminated by the addition of 750 µl $Na_2S_2O_5$ and 500 µl KI as a carrier.

A column (12 x 1 cm) of Sephadex G50 was prepared as described previously and the contents of the reaction vessel transferred to the column and eluted with buffer into 1 ml fractions (63 x 11 mm polystyrene tubes containing 0.1 ml 5% BSA in 0.01 M PBS). A typical elution profile is given in Table 2.2. In this instance labelled hormone was eluted in fraction 6 and the free ^{125}I in fractions 13-14. The labelled hormone (specific activity 60-120 mCi/mg) was stored at 4°C and used within 2-3 weeks of preparation.

2.3 RADIOIMMUNOASSAY OF PROTEIN HORMONES

For a number of experiments described in Chapters 4 and 5, serum concentrations and pituitary content of LH and FSH were assayed. The methods used were essentially those described by Fraser & Sandow, 1977 and de Jong & Sharpe, 1977. Some of the assays were performed by Mrs. M. Swaney.

Reagents: Iodinated rat LH (prepared as described)

Iodinated rat FSH (kindly prepared by Mrs. J. Smith by Chloramine T iodination)

Assay buffer 0.01 M PBS, 0.01% thiomersalate, 1% BSA pH 7.5

Special buffer 0.01 M PBS, 0.01% thiomersalate, 0.05 M

ethylenedieminetetraacetic acid (EDTA)

1% BSA

Normal rabbit serum (NRS) Wellcome

Standards Rat LH-RP-1 NIAMDD, NIH, U.S.A.,

Rat FSH-RP-1 NIAMDD, NIH, U.S.A.,

Antisera Anti rat LH-S-4 from NIAMDD

Anti ovine FSH (619II) from Dr. J.M.J. Uilenbroek

Second antibody Donkey-anti-rabbit serum (DARS) Wellcome.

LH Assay Procedure

All additions were made using an automatic dispensing system (Microlab M) into 63 x 11 mm polystyrene tubes. All incubations were carried out at 4°C.

Day 1 Duplicate estimations of non-specific binding (NSB), total binding (Bo), standards (serial dilutions from 0.7-100 ng/0.1 ml) and samples were made to a total volume of 0.4 ml. Subsequent additions were as follows:-
0.1 ml of antiserum (1:8,000 in special buffer).

Day 2 0.1 ml ^{125}I-LH in assay buffer (15,000 cpm).

Day 3 0.1 ml (1:15 dilution) DARS.

Day 4 Separation of free from bound hormone was achieved after dilution with 1 ml saline followed by centrifugation at 3000 rpm for 30 minutes at 4°C.
The pellet was counted and the assay calculated using a gamma spectrometer attached to a commodore 4032 computer (Nuclear Enterprises).

The assay sensitivity was 6 ng/ml and the within assay coefficient of variation 9%.

FSH Assay Procedure

Assay conditions were identical to those for LH except that standards ranged from 3.9-500 ng/0.1 ml and the total assay volume was 0.5 ml.

FSH Antiserum was used at 1:8,000 dilution. The assay sensitivity was 50 ng/ml and the within-assay coefficient of variation was 11%.

Prolactin

Prolactin concentrations were estimated for an experiment described in Chapter 4 section 4.3.2.2. These samples were assayed by Dr. A.S. McNeilly (McNeilly et al., 1978).

2.4 RADIOIMMUNOASSAY OF PEPTIDE HORMONES

Hypothalamic content of LHRH was assessed in Chapter 4, experiment 4.4.3.

Radioimmunoassays for LHRH and LHRH agonist were utilized for experiments described in Chapter 8.

Reagents: Iodinated LHRH agonist (prepared as described previously)

Iodinated LHRH

Standards 0.39-400 pg/0.1 ml LHRH agonist (Hoechst A.G.)

0.39-400 pg/0.1 ml LHRH

Assay buffer 0.01 M PBS, 0.01% thiomersalate, 0.1% BSA pH 7.4

Antisera 1. R103 anti-LHRH agonist at 1 in 4,000

dilution (Fraser et al., 1983a)

2. RO "C-terminal" anti-LHRH at 1 in 15,000 dilution (Jeffcoate, 1976)

3. R42 "conformational" anti-LHRH at 1 in 30,000 dilution (Nett et al., 1973).

Assay Procedure

All additions were made using an automatic dispenser system (Microlab M) into 63 x 11 mm polystyrene tubes. Incubations were at 4°C.

Day 1 Duplicate determinations of NSB, Bo, sample or standards were made in 0.2 ml total volume, together with 50 µl of the relevant antiserum at the appropriate dilution.

Day 2 Addition of 50 µl labelled hormone (15,000 cpm)

Day 3 Separation of free from bound was achieved at 4°C by addition of 1.5 ml ice cold ethanol followed by centrifugation at 2500 rpm for 20 minutes at 4°C. The pellet was counted and the assays calculated as described for LH.

The detection limit for each assay was 5-8 pg and the within assay coefficient of variation was 9-11%.

Hypothalamic content of LHRH was assessed by RIA after extraction as described.

Method

Hypothalami were rapidly dissected out after death and placed in 1 ml boiling PBS for 10 minutes. Samples were stored at -40°C until required for assay. The contents were thawed and homogenized in a 1 ml glass homogenizer. The homogenate was transferred to plastic 63 x 11 mm tubes together with 1 ml 0.2% BSA PBS which had rinsed out the homogenizer. After centrifugation at 3,000 rpm for 60 minutes at 4°C the supernatant was taken for radioimmunoassay as described.

2.5 RADIOIMMUNOASSAY OF STEROID HORMONES

For the in vitro experiments described in Chapter 7, steroid hormones were assayed from unextracted culture medium, no difference having been found between extracted and unextracted medium.

For experiments where serum steroid concentrations were estimated (Chapters 4 and 5) the following procedure was used prior to assay.

Extraction of steroids from serum

Estimation of individual recoveries was obtained by incubating sample aliquots with 1000 cpm of internal standard (^{3}H-steroid) for 30-60 minutes in 125 x 15 mm glass tubes. The extraction procedures differed for each steroid:-

Testosterone was extracted using 10 volumes of freshly distilled hexone : diethyl ether (4 : 1 v/v)

Progesterone and oestradiol were extracted using 2 mls petroleum ether

After vigorous mechanical shaking for 5 minutes using a multi-vortex shaker (Searle) the organic and aqueous phases were separated by freezing in a dry ice/methanol mixture. The organic phase was decanted and evaporated to dryness on a heated block and

the residue reconstituted in 200 µl assay buffer and left for at least 3 h prior to estimation of tracer recovery and assay.

Assay Reagents

Assay buffer — phosphate gelatin buffered saline (PGBS) pH 6.8

0.9% NaCl

0.86% $Na_2 HPO_4$

0.61% $NaH_2PO_4\ 2H_2O$

0.1% Gelatin

0.01% thiomersalate

Scintillation fluid — 10 g 2,5-diphenyloxazole

0.75 g 1,4-Di[2-(5-phenyloxazolyl)] benzene from Koch Light Labs, dissolved in 2.5 litres sulphur free toluene (A.&.J. Beveridge) and 1.5 litres Triton-X (A.&.J. Beveridge).

Tracer hormones - were all stored at 4°C in ethanol.

[1,2,6,7 ^{3}H (N)] testosterone)New England

[1,2,6,7 ^{3}H (N)] progesterone)Nuclear,

Androst-4-Ene-3,17,Dione[1B,2B ^{3}H(N)])Boston Mass

[2,4,6,7 ^{3}H] oestradiol (Radiochemicals, Amersham).

Standards

Non-radioactive steroids (Sigma Chemical Co.) were dissolved in ethanol and stored at 4°C at 1 mg/ml stock solution. Standard solutions were either made (a) in ethanol at 10 ng and 1 ng/ml (e.g. oestradiol) and the standard cure obtained by pipetting the ethanol

and, after drying down, taking up the residue in 0.1 ml PGBS; or (b) from the stock solution and stored in PGBS at 4°C (e.g. testosterone 10-640 pg/0.1 ml; androstenedione 10-1000 pg/0.1 ml and progesterone 10-800 pg/0.1 ml).

Antisera

1. Testosterone: Antiserum E01 (kindly donated by Dr. S. Tillson) was used at a dilution of 1 in 10,000 giving 50-55% specific binding (Corker & Davidson, 1978).

2. Progesterone: Antiserum 361 was used at a dilution of 1 in 4,000 giving 40-50% specific binding (Scaramuzzi et al., 1975).

3. Androstenedione: Antiserum IR637 was used at a dilution of 1 in 8,000 giving 40-45% specific binding (Vaughan-Williams et al., 1982).

4. Oestradiol: Antiserum R3 was kindly donated by Dr. H. Dobson and utilized for assay of culture medium at a dilution of 1 in 8,000 giving 40-45% specific binding (Van Look et al., 1977).

Serum oestradiol levels were measured, after plasma extraction, utilizing a gamma G antibody (Bäckstrom et al., 1982) and were assayed by Mrs. M. Swaney.

The lower detection limits of the assays were 10 pg/0.1 ml for androstenedione and progesterone and 5 pg/0.1 ml for testosterone and oestradiol (R3). The detection limit for serum oestradiol assay was 1 pg/0.1 ml.

The within-assay variation was 5-9% and the interassay variation 10-15%. All samples from one experiment were assayed at the same time.

Assay Procedure

Duplicate estimations of NSB, Bo, standards or samples (in 0.1 ml) were made. Samples were dispensed into 75 x 10 mm glass tubes using a Microlab M automatic dispenser. 100 µl of antiserum (made up to the appropriate dilution in PGBS) was added, followed by 100 µl tracer (6-8,000 cpm). After thorough mixing the assay was left for 12-24 h at 4°C.

Separation of free from bound hormone was achieved on ice using 1 ml freshly made cold dextran coated charcoal (0.25% charcoal - Sigma Chemicals, 0.025% Dextran T70, Pharmacia, in PGBS). Charcoal addition was completed within 2-3 minutes and the tubes vortexed and left for 15 minutes at 4°C prior to centrifugation at 2000 rpm for 15 minutes at 4°C. The supernatant (antibody bound hormone) was then poured off into 10 mls of Scintillation fluid. Radioactivity was measured by counting each vial for 4-5 minutes in a β counter (Packard, counting efficiency 50%).

2.6 IMMUNIZATION

Rats were actively immunized against LHRH, LH and testosterone (Chapter 4).

In order for small molecules such as testosterone or LHRH to be rendered immunogenic it is necessary to couple the molecule to a large molecular weight carrier such as serum albumin.

Testosterone was conjugated to BSA at position 15 (Rao & Moore, 1976) by Dr. J.E.T. Corrie.

LHRH was coupled to human serum albumin (HSA) by carbodiimide as the coupling agent (Fraser et al., 1974).

Immunization procedure for LHRH

Day 1 To prepare conjugate to immunize 10 rats, 5 mg HSA (Sigma) were combined with 5 mg LHRH and dissolved in 0.7 ml distilled water. 100 mg carbodiimide (Sigma) was dissolved in 0.3 ml distilled water and immediately added to the HSA-LHRH solution, mixed and left in the dark overnight at room temperature.

Day 2 Six inches of dialysis tubing was washed in distilled water and knotted at one end before the conjugate mixture was added. The top was then knotted and the sac left to dialyse for 48 h at 4°C against 0.9% saline. Since the dialysis tubing cut off was at Mr 10,000 and LHRH Mr is approximately 1000 and BSA 50,000, uncoupled LHRH molecules will pass through the tubing.

Day 4 The contents of the dialysis tubing were then combined with Freund's complete adjuvant (Gibco Labs, Grand Island Biological Centre, Grand Island, New York, U.S.A.) in a ratio of 2:3. An emulsion was then formed by mechanical means using glass 10 ml syringes (Berlin & McKinney, 1958).

LH is immunogenic without conjugation and was therefore dissolved in distilled water (100 µg/rat) before emulsification in Freund's complete adjuvant as described.

Animals were immunized by intradermal injections (given under ether anaesthesia) of 1 ml emulsion distributed between 8-10 sites.

Booster immunizations were given 3-6 months after the initial injection utilizing Freund's incomplete adjuvant.

2.7 ANTIBODY TITRE DETERMINATION

Antibody titres for testosterone and LHRH were assessed 3-4 months after immunization (Chapter 4).

The titre of anti-LHRH or anti-testosterone antibodies was determined by incubation of serial dilutions of serum together with 10,000 cpm ^{125}I-labelled LHRH or [^{3}H]-testosterone using conditions similar to those described for radioimmunoassay. In each case the dilution of serum required to bind 33% of the labelled hormone was taken as the antibody titre.

2.8 DNA AND PROTEIN ASSAYS

Protein estimations were assessed using the method of Lowry *et al.*, 1951 with BSA as a standard.

DNA measurements were performed according to the method of Burton, 1956 and were carried out by Mr. G. Menzies.

2.9 STATISTICAL ANALYSIS

The results obtained in this thesis were analysed using standard statistical procedures and are expressed as mean $\pm$ S.E.M. The significance of differences between means has been assessed using Student's t-test or (where appropriate) two factor analysis of variance with replication. These were performed using programmes written for a Hewlett-Packard desk top calculator (Model 9821A) by Dr. R. Sharpe

CHAPTER 3

LHRH RECEPTORS - CHARACTERIZATION AND MEASUREMENT

CHAPTER 3

LHRH RECEPTORS - CHARACTERIZATION AND MEASUREMENT

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3.3 BIOCHEMICAL NATURE OF LHRH RECEPTORS

3.1 PITUITARY LHRH RECEPTORS

3.1.1 Introduction

Although the pituitary gland was known to be the target organ for hypothalamic LHRH, the demonstration of specific high affinity receptors proved difficult. Initial studies successfully utilized iodinated (^{125}I-LHRH e.g. Spona, 1973; Marshall *et al.*, 1976; Heber *et al.*, 1978) or tritiated (^{3}H-LHRH e.g. Grant *et al.*, 1973; Theoleyre *et al.*, 1976) LHRH to demonstrate the presence of specific pituitary binding sites for LHRH. Analysis of the binding characteristics, as described by Scatchard plot analysis, indicated the presence of two separate binding sites, one of low affinity (Ka of 10^{5}-10^{6} M^{-1}) but high capacity and the other of high affinity (Ka 10^{8}-10^{9} M^{-1}) but low capacity. This was observed in both rat (Marshall *et al.*, 1976; Heber *et al.*, 1978; Clayton *et al.*, 1978) and bovine (Marshall *et al.*, 1976) pituitary membrane preparations.

Although the high affinity sites represented only a small percentage of the total bound hormone (e.g. Clayton *et al.*, 1979b) their affinity was of the range expected from estimations of portal blood LHRH (40-800 pM). It was suggested therefore that the high affinity site represented the physiological receptor whereas the low affinity site represented a membrane-associated degrading enzyme (Clayton *et al.*, 1979b). Thus if degradation of ^{125}I-LHRH was prevented by a peptidase inhibitor aprotinin, a single class of high affinity receptors was detected (Wagner *et al.*, 1979).

However, with the availability of agonist analogues of LHRH relatively resistant to degradation (e.g. Swift & Crighton, 1979) the

need for the inclusion of protease inhibitors has been largely removed. A major advance in the analysis of LHRH receptors was the advent of ^{125}I-LHRH agonist analogues which were found to bind primarily to the high affinity site (Conne et al., 1979), with only 30% degradation occurring after 30 minutes at 37°C (Clayton et al., 1979b). The physiological significance of these receptors has been indicated by the correlation between the relative potencies of ^{125}I-agonists and their biological activity, and the ability of unlabelled LHRH to displace binding of ^{125}I-LHRH agonist (e.g. see Clayton & Catt, 1981b).

It is important to remember, however, that the ^{125}I-agonist or antagonist analogues used in receptor assays are only tools to detect the high affinity binding sites. The endogenous ligand is subject to rapid degradation by peptidases (possibly represented by the low affinity binding site). Therefore control of the rate of ligand degradation is likely to play an important role in the control of target organ sensitivity.

3.1.2 Aims

The aim of this study was to develop a radioreceptor assay capable of measuring pituitary LHRH receptor concentrations in individual pituitary glands, to enable subsequent characterization of the factors controlling receptor numbers and to assess their importance in the regulation of pituitary sensitivity (Chapter 4).

3.1.3 Assessment of LHRH Receptors in individual pituitaries

The method adopted was essentially the 'saturation analysis method' reported by Clayton, et al., 1980.

3.1.3.1 Animals

Unless otherwise stated all the rats utilized in this thesis were of the Sprague Dawley strain, bred in the laboratory colony and kept under standard conditions (lights on 05.00 h - 19.00 h and with free access to food and water). Animals were killed using dry ice CO_2 and the anterior pituitary rapidly dissected out following decapitation.

3.1.3.2 Method

Individual anterior pituitaries were assessed for LHRH receptor content immediately after removal from the animal, by homogenization on ice in 400 µl 10 mM Tris HCl (pH 7.4) in a 1 ml glass homogenizer. The homogenate was then filtered through nylon gauze (Simon Precision textiles, Stockport, Cheshire, U.S., Mesh type N355). Duplicate 50 µl aliquots of the filtrate were equilibrated with 100,000 - 150,000 cpm ^{125}I-LHRH agonist plus 420 pg unlabelled hormone, giving a total ligand concentration of 540-600 pg (2.2-2.4 nM). Non-specific binding (NSB) was assessed in duplicate in the presence of 10^{-5} M unlabelled LHRH agonist. Incubations were carried out for 80 minutes at 4°C (conditions found to be optimum for maximal binding). The reaction was terminated by dilution with 2 mls ice cold 0.01 M phosphate buffered saline (PBS) followed by immediate filtration under vacuum through Whatman GF/C glass fibre filters (which had been presoaked for 12 h in 2% BSA to minimize non-specific tracer adsorption). After washing with 3 x 2 ml PBS, the dried filters were counted for estimation of the retained hormone receptor complex. Specific binding (Bo) was assessed by subtracting the NSB value (2-3% of total counts added) from the total bound (B).

The protein content of pituitary glands as assessed by the method of Lowry et al., 1951, ranged from 1.4 - 2 mg. However, since expression of the results as femtomoles (fm) bound per mg protein made no significant differences to the relative changes in receptor numbers seen when the results were expressed as fm/pituitary, the latter format was adopted for presentation of pituitary receptor concentrations. In addition no significant differences were found between receptor numbers assessed from fresh or frozen tissue.

3.1.3.3 Calculation of pituitary LHRH receptor concentration

The bindable activity of the tracer utilized in the receptor studies calculated from the Leydig cell self displacement curve (Sharpe & Fraser, 1980a) was 1.2 pg per 1000 cpm. The counts of LHRH agonist specifically bound (B - NSB) divided by the total amount of LHRH added (i.e. hot and cold) gave a measure of the pg bound/50 µl homogenate. This value was then multiplied by 8 and calculated as fm bound/gland.

Since a ligand concentration of 1.8 - 2 nM had been shown to produce about 85% receptor occupancy (Clayton, 1980) it is assumed that the binding analysis was performed under near saturating conditions (i.e. in the presence of 2.2-2.4 nM ligand) and therefore that specific binding is a direct measure of the number of receptors per aliquot.

This method of calculating receptor numbers has been criticised since it does not enable measurement of possible changes in receptor affinity (Ka). Thus, some studies estimate receptor concentrations, together with Ka, from competition curves and Scatchard analysis. However, it is necessary to utilize pooled homogenates from a number

of pituitaries to obtain such data, and the precise determination of individual pituitary receptors is therefore lost. In addition, similar changes in receptor concentrations have been observed by both methods (e.g. Savoy-Moore et al., 1980; Clayton et al., 1980) and no evidence has been found to suggest changes in pituitary LHRH receptor affinity under any endocrine state so far studied. The technique of saturation analysis was thus utilizied in the assessment of pituitary LHRH receptors, for the studies presented in Chapter 4.

3.2 OVARIAN LHRH RECEPTORS

3.2.1 Introduction

Initial binding studies utilizing ^{125}I-LHRH revealed the presence of specific, but low affinity binding sites in the ovary similar to those found in the pituitary (Marshall et al., 1976; Heber et al., 1978). In addition ovarian uptake of ^{125}I-LHRH agonist in vivo implicated the presence of specific binding sites (Mayar et al., 1979). The first conclusive demonstration of high affinity ovarian LHRH receptors was obtained by incubating labelled LHRH agonist with homogenates from ovaries of PMSG/hCG-primed immature rats (Clayton et al., 1979a). The receptors had identical properties both of affinity (Ka 5 x 10^9 M^{-1}) and specificity to those of the pituitary, although their concentration was markedly lower (Clayton et al., 1979a).

3.2.2 Aims

The aims of these investigations were threefold:-

1) To characterize and develop conditions suitable for analysis of LHRH receptors in rat follicular tissue with a view to utilizing the

radioreceptor assay for the detection of intragonadal LHRH-like peptides.

2) To develop a method suitable for the estimation of ovarian LHRH receptor concentrations in individual ovaries.

3) To identify the sites of LHRH binding in ovarian tissue by autoradiography.

3.2.3 LHRH binding to rat follicular tissue

Since direct effects of LHRH had been shown on follicular as well as luteal tissue (Chapter 1) and only the receptors on luteal tissue had been adequately characterized, it was decided to investigate ovarian LHRH receptors from PMSG-primed immature rats.

3.2.3.1 Animals and Method

Animals

Immature (26-30 days old) female Sprague Dawley rats from the laboratory colony were used for these studies. The rats were killed using dry ice CO_2 48 h after induction of follicular development by subcutaneous injection of 50 IU Pregnant Mares Serum Gonadotrophin (PMSG) and the ovaries rapidly dissected out into ice cold 10 mM Tris HCl (pH 7.5) 0.5 ml/ovary. After homogenization in a 10 ml glass homogenizer the homogenate was filtered through nylon gauze as described for pituitary tissue.

Method

150 µl aliquots of filtrate (600-700 µg protein) were used for binding assay which was carried out in a total volume of 400 µl of assay buffer (10 mM Tris HCl, 0.2% BSA). Total binding was assessed after incubation in the presence of 100,000 - 120,000 cpm ^{125}I-LHRH agonist, non-specific binding being estimated in

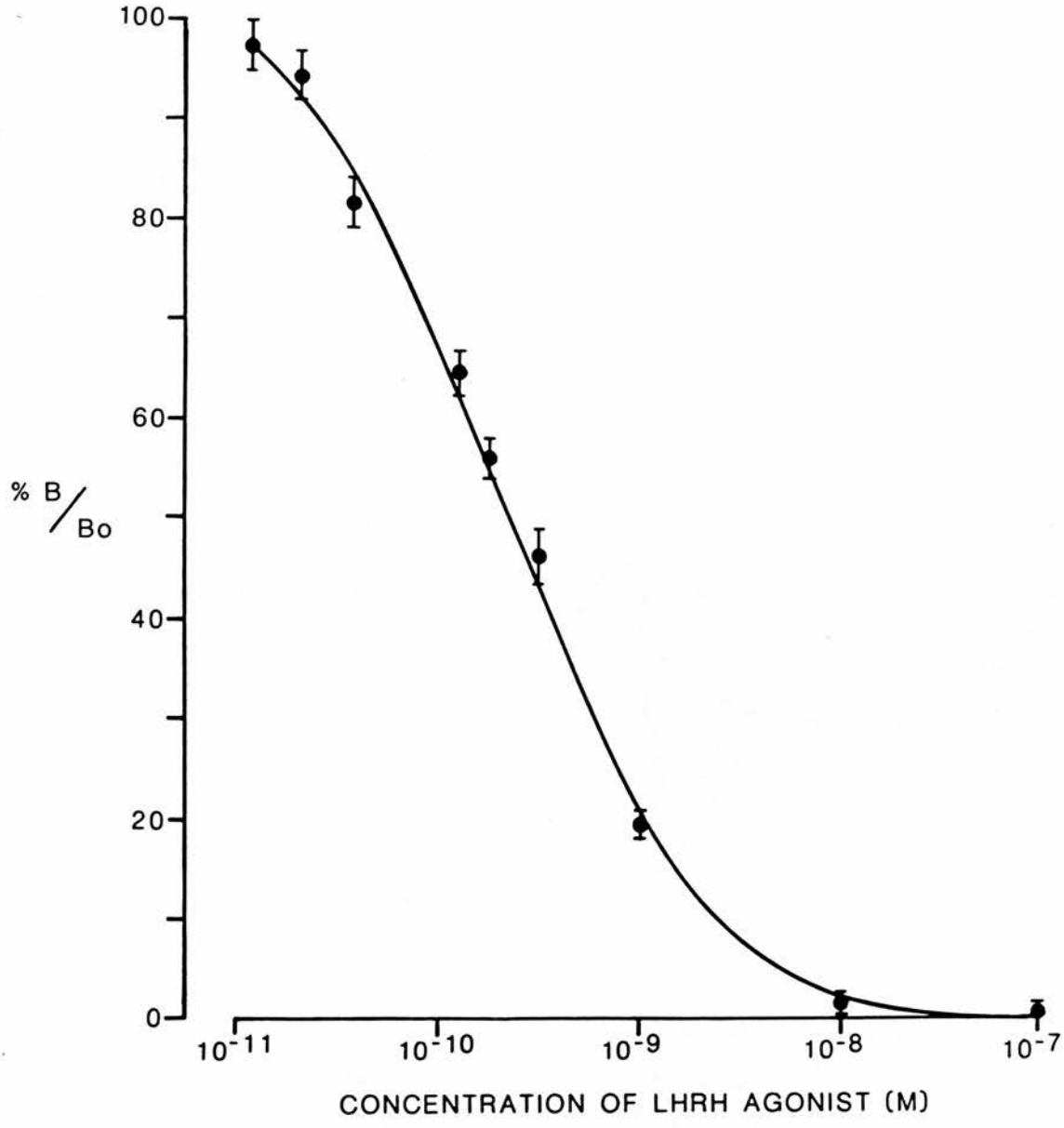

FIGURE 3.1 Displacement of ^{125}I-LHRH agonist binding to rat ovarian homogenates by unlabelled LHRH agonist. Each point represents mean ± range of duplicate determinations.

duplicate in the presence of 10^{-5} M unlabelled hormone.

3.2.3.2 Separation of free from bound

This was achieved by one of two methods:-

3.2.3.2.1 Filtration

After incubation, 1 ml of ice cold PBS was added to each tube and the contents immediately filtered through Whatman GF/C filters, using the conditions described earlier for the pituitary (3.1.3.2). In practice, pre-soaking the filters in 2% BSA reduced non-specific binding of the tracer from 5-10% of the total counts to 3% and this figure was reduced to less than 1% following the washing procedures.

Using this method NSB values were 2-4% of total counts and specific binding was 10-15% per mg protein. This is a much lower value than that obtained with pituitary tissue (30-40%/mg protein), in agreement with the lower concentration of receptors in ovarian tissue.

A displacement curve was obtained by incubation of ovarian tissue in the presence of a constant amount of labelled hormone with varying amounts of unlabelled LHRH agonist (Fig. 3.1). The curve was found to be identical to that obtained with pituitary tissue (Chapter 2, Fig. 2.2), both binding sites showing 50% displacement of binding with 3×10^{-10} M LHRH agonist.

3.2.3.2.2 PEG/IgG Separation

Separation of free from bound hormone using solubilized hCG receptors can be achieved by a double precipitation procedure with polyethylene glycol (PEG) in the presence of bovine gamma globulin (IgG) as a carrier (Dufau et al., 1973). This technique has been

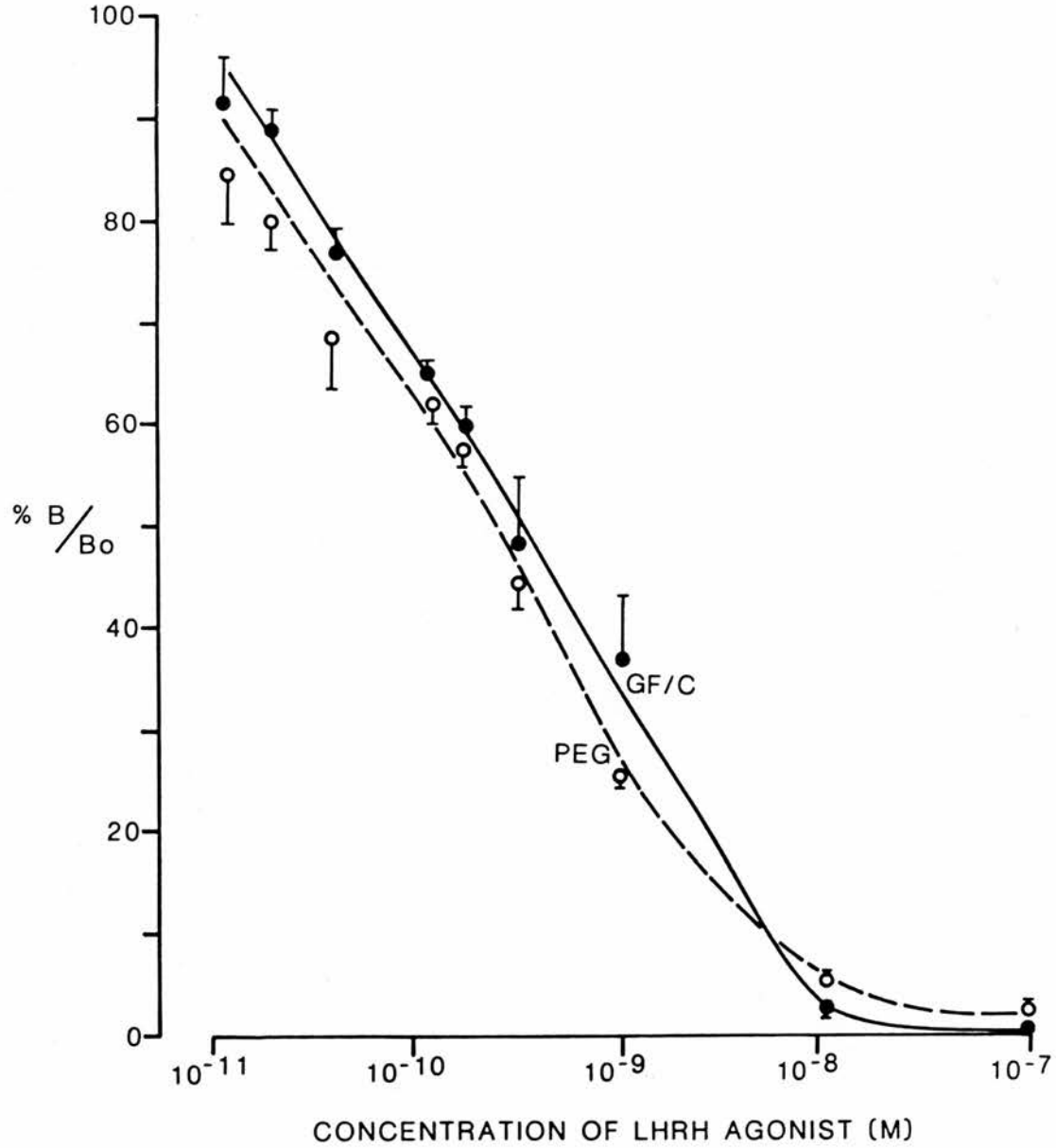

FIGURE 3.2 Displacement of ^{125}I-LHRH agonist binding to rat ovarian tissue by unlabelled LHRH agonist following separation of free from bound by polyethylene glycol precipitation (o--o PEG) or filtration through Whatman GF/C filters (GF/C •—•). Each point represents mean ± range of duplicate determinations.

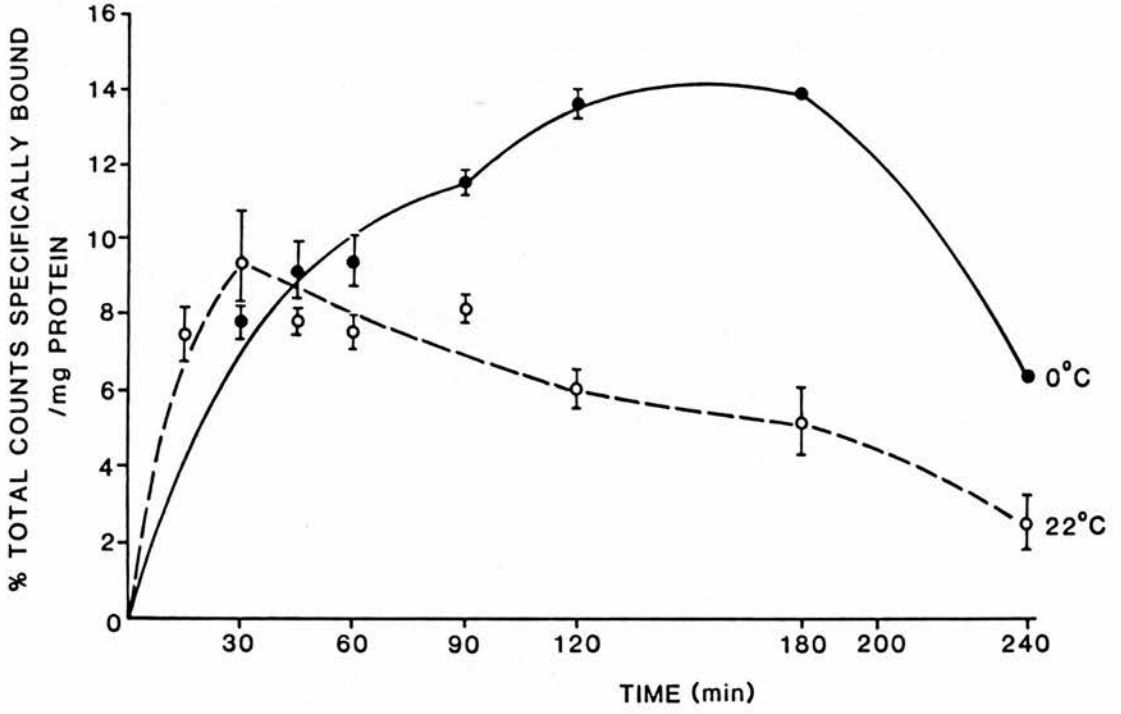

FIGURE 3.3 Time course of specific binding of ^{125}I-LHRH agonist to rat ovarian tissue at 0°C or 22°C. Each point represents mean ± range of duplicate determinations.

utilized for other peptide hormones e.g. insulin (Desbuquois & Aurbach, 1971) and was suggested as being particularly useful for low molecular weight substances.

For LHRH receptors, after incubation as described above, the tubes were placed on ice and diluted with 0.5 ml cold PBS followed by 0.5 ml 0.5% bovine IgG (Bovine Cohn Fraction II, Sigma Chemicals) and 1 ml 25% PEG (w/v) (Approx. Mr. 8000, Sigma Chemicals). After thorough mixing the tubes were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was aspirated and the pellet counted. A blank value of 2-3% of total counts was obtained and an NSB of 4-6%. Total specific binding varied between 15-20%.

A comparison of the two methods of separation is shown in Fig. 3.2. The displacement curves obtained were not significantly different so it was concluded that either method was suitable for the separation of free from bound LHRH agonist.

3.2.3.3 Time and temperature dependence of LHRH agonist binding

Optimal conditions for assay incubation were determined by assessment of specific binding, as described previously, at 0°C or 22°C for various times up to 240 minutes (Fig. 3.3). Binding was rapid at 20°C, reaching maximal levels after 30 minutes. Binding then decreased with increased incubation time at 20°C, a phenomenon likely to be due to instability of the ligand and/or receptor under these conditions. At 0°C binding reached maximal levels after 120 minutes and was stable for 180 minutes.

Assessment of ovarian LHRH receptors was therefore routinely achieved after incubation for 120 minutes at 0-4°C.

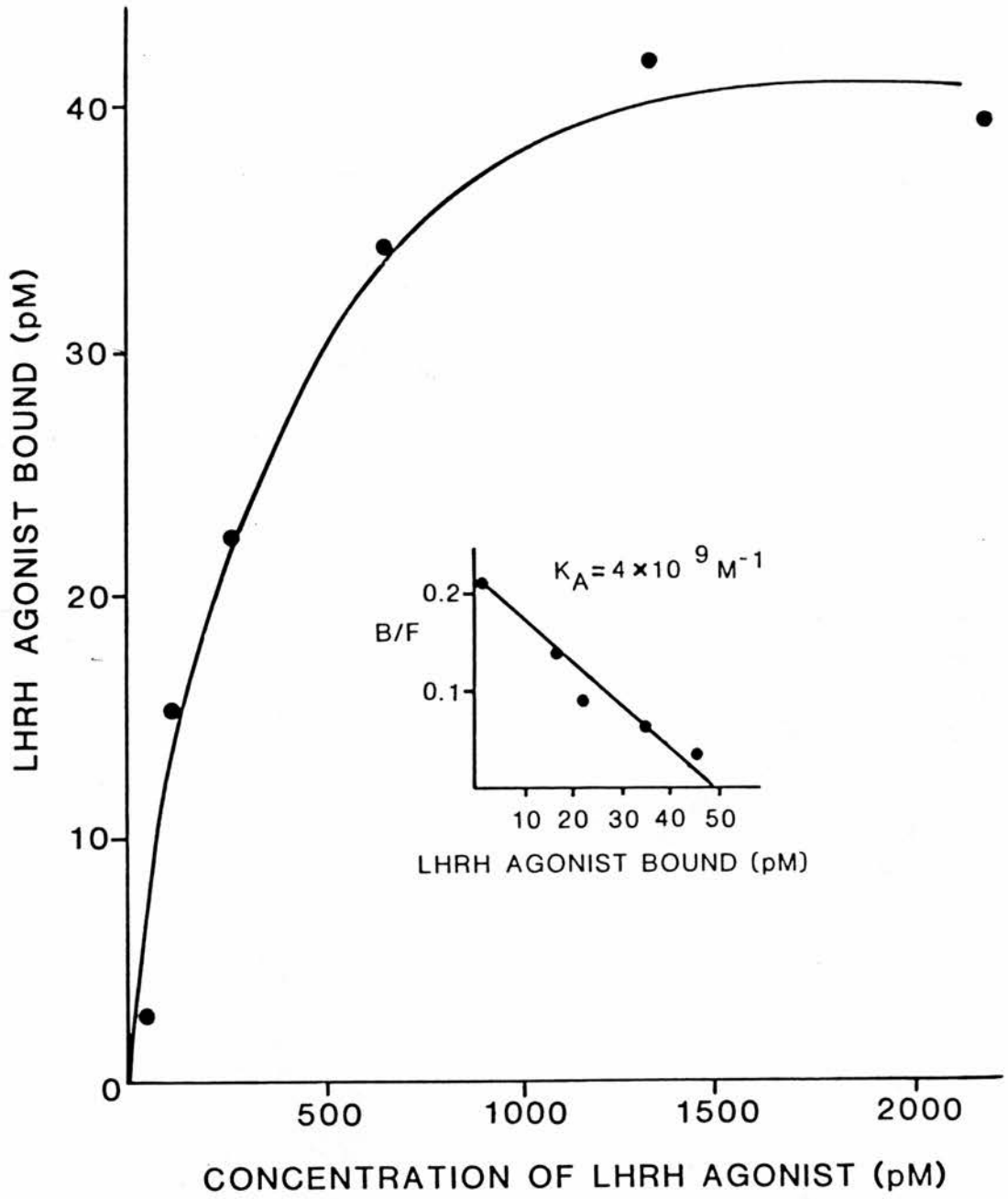

FIGURE 3.4. Saturation of ^{125}I-LHRH agonist binding to rat ovarian tissue with a Scatchard plot of the data as inset. Each point represents the mean of duplicate determinations. The within assay variation for the duplicates was 10%. Over 10 ovarian receptor assays the intra-assay coefficient of variation was 13%.

3.2.3.4 Scatchard analysis of ^{125}I-LHRH agonist binding

Aliquots of tissue were incubated, as described above, with increasing concentrations of ^{125}I-LHRH agonist. Plotting the data as bound versus added hormone gave a curvilinear plot indicative of saturable binding sites (Fig. 3.4). Expression of the data as the ratio of bound to free hormone (B/F) against bound (B) hormone resulted in a linear Scatchard plot indicative of a single class of receptors with affinity Ka 4 x 10^9 M^{-1} (Fig. 3.4).

Although Scatchard analysis was designed to characterize low affinity binding systems interacting with small soluble molecules (Scatchard, 1949) and is therefore not ideal for using in the analysis of hormone-receptor interactions, it is universally used as an index of receptor affinity. The apparent affinity of ^{125}I-LHRH agonist for rat follicular receptors is in agreement with the value obtained for both rat luteal (Clayton et al., 1979a) and pituitary tissue (e.g. Clayton & Catt, 1981b).

Interestingly, whereas the pituitary receptor Ka is greater than the ED50 value for LH release (which has been suggested to indicate that a maximum biological response can be achieved with only a fraction of the receptors occupied (Clayton & Catt, 1981b)) the Ka and ED50 for ovarian LHRH receptors and direct actions are of the same order of magnitude (10^{-9} M).

3.2.3.5 Specificity of ovarian LHRH receptors

The ability of ovarian LHRH receptors to discriminate between LHRH and unrelated peptides was assessed by performing a binding assay with ^{125}I-LHRH agonist as the labelled ligand in the presence of various concentrations of LHRH agonist, LHRH, TRH,

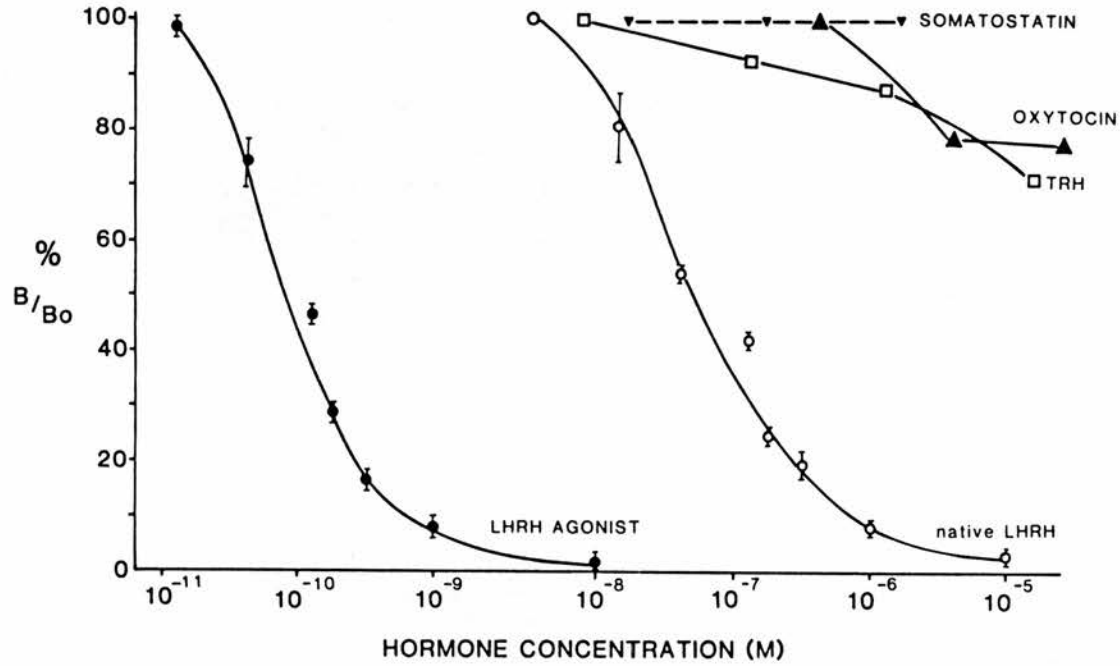

FIGURE 3.5 Displacement of ^{125}I-LHRH agonist binding to rat ovarian tissue by unlabelled LHRH agonist (●—●), LHRH (○—○), Somatostatin (▼- -▼), oxytocin (▲—▲) and TRH (□—□)

oxytocin and vasopressin; using the conditions described earlier.

The displacement of binding of ^{125}I-LHRH agonist obtained with each peptide is shown in Fig. 3.5. Native LHRH was capable of displacing binding but the curve was shifted to the right in comparison with that of unlabelled LHRH agonist. The lower affinity of the receptor for LHRH is probably due to its degradation during incubation. Somatostatin had no effect on binding and TRH and oxytocin only produced minimal displacement at high concentrations.

The receptor data again emphasises the similarity between the ovarian and pituitary receptor and is corroborated by biological data. TRH and somotostatin had no effect *in vitro* in isolated follicles (Chapter 7).

3.2.3.6 Conclusions

The criteria laid down for the designation of a "physiological receptor" (Cuatrecasas, 1974 & Cuatrecasas *et al.*, 1975) have largely been confirmed for LHRH receptors. Binding is (a) tissue specific for both the pituitary and gonads, (b) ligand specific, only LHRH and related compounds being capable of receptor binding (Fig. 3.5), (c) of high affinity (Fig. 3.4), being within the range known to initiate biological responses (Chapter 7), (d) reversable (Clayton & Catt, 1981b) and (e) saturable (Fig. 3.4).

3.2.4. Assessment of LHRH Receptors in individual ovaries

The concentration of LHRH receptors present in homogenates of whole ovarian tissue was assessed by saturation analysis using essentially the same method as that described for the pituitary (3.1.3). The ovarian assay was incubated for 120 minutes at 4°C and terminated by filtration through Whatman GF/C filters. Since ovarian

protein content varied considerably between treatment groups, according to ovarian size, it was decided to express the receptor data in terms of fm bound/mg protein rather than fm bound/ovary. Since ovarian receptors are believed to recognize locally produced factors (Chapter 1) it is likely that receptor concentration rather than total number per ovary is the more important parameter. However, where major differences were obtained by expressing the data as a concentration, both results are shown (Chapter 5).

Although no attempt was made to assess changes in receptor affinity, other groups had failed to show this (e.g. Pieper et al., 1981; Reeves et al., 1982) and it was concluded that ovarian LHRH receptor affinity was unlikely to be altered and therefore that saturation analysis provided an adequate means of assessing receptor regulation.

3.2.5 Localization of LHRH binding by autoradiography

3.2.5.1 Introduction

Relatively few studies have been reported as determining the cellular location of ovarian LHRH receptors. Although receptors had been demonstrated in isolated granulosa cells (Jones et al., 1980; Ranta et al., 1982) and luteal tissue (Clayton et al., 1979a; Pieper et al., 1981) the possibility of binding to other tissue compartments had not been established. The aim of this investigation was to compare the localization of LHRH binding by autoradiography in both pituitary and ovarian tissue, with particular emphasis on whether LHRH binding occurred to thecal tissue.

3.2.5.2 Method

Two pro-oestrous rats weighing 250 g were anaesthetized at 12.00

h with ethyl carbamate (1.75 g/Kg body wt, injected i.p. as a 25% solution (w/v) BDH Ltd., Poole, Dorset, U.K.) and the right atrium of each was cannulated. Each rat was given a 1 ml injection of ^{125}I-labelled LH-RH agonist (2.9 x 10^7 cpm) either alone or with a 100 fold excess of unlabelled hormone at pH 7, into the atrial cannula over a period of 2 minutes. The animals were killed 30 minutes later by perfusion with 30 ml heparinized saline and this was immediately followed by 20 ml 10% neutral buffered formal-saline solution. The ovaries and pituitary glands were dissected out, immersed in this fixative solution and counted in a gamma spectrometer. In the animal injected with ^{125}I-labelled LH-RH agonist the ovary count was 2100 cpm/mg, the pituitary 3200 cpm/mg and the blood count 270,000 cpm/ml. The corresponding values for the rat injected with ^{125}I-labelled LH-RH agonist and unlabelled hormone were 100 cpm/mg and 350 and 220,000 cpm/ml.

After fixation for 4 h the tissues were transferred to 2% glutaraldehyde (TAAB, Reading, Berks, U.S.) in 0.01 M Hepes Buffer (pH 7.2) for further fixation overnight at 4°C. The tissues were then washed with distilled water, snap frozen and sectioned at 12 µm with a cryostat. The sections were mounted on acid-cleaned and gelatin-coated slides and coated with liquid nuclear emulsion (K5, Ilford Ltd., Basildon, Essex, U.K.). Additional tissues from untreated rats were similarly prepared to control for chemographic effects (high background activity and latent image fading). The slides were exposed in light-proof boxes at 4°C for 33 days. They were then developed and fixed (D19 developer and Kodafix, Kodak Ltd., Liverpool, U.K.) and stained with haemalum and eosin. Each slide was

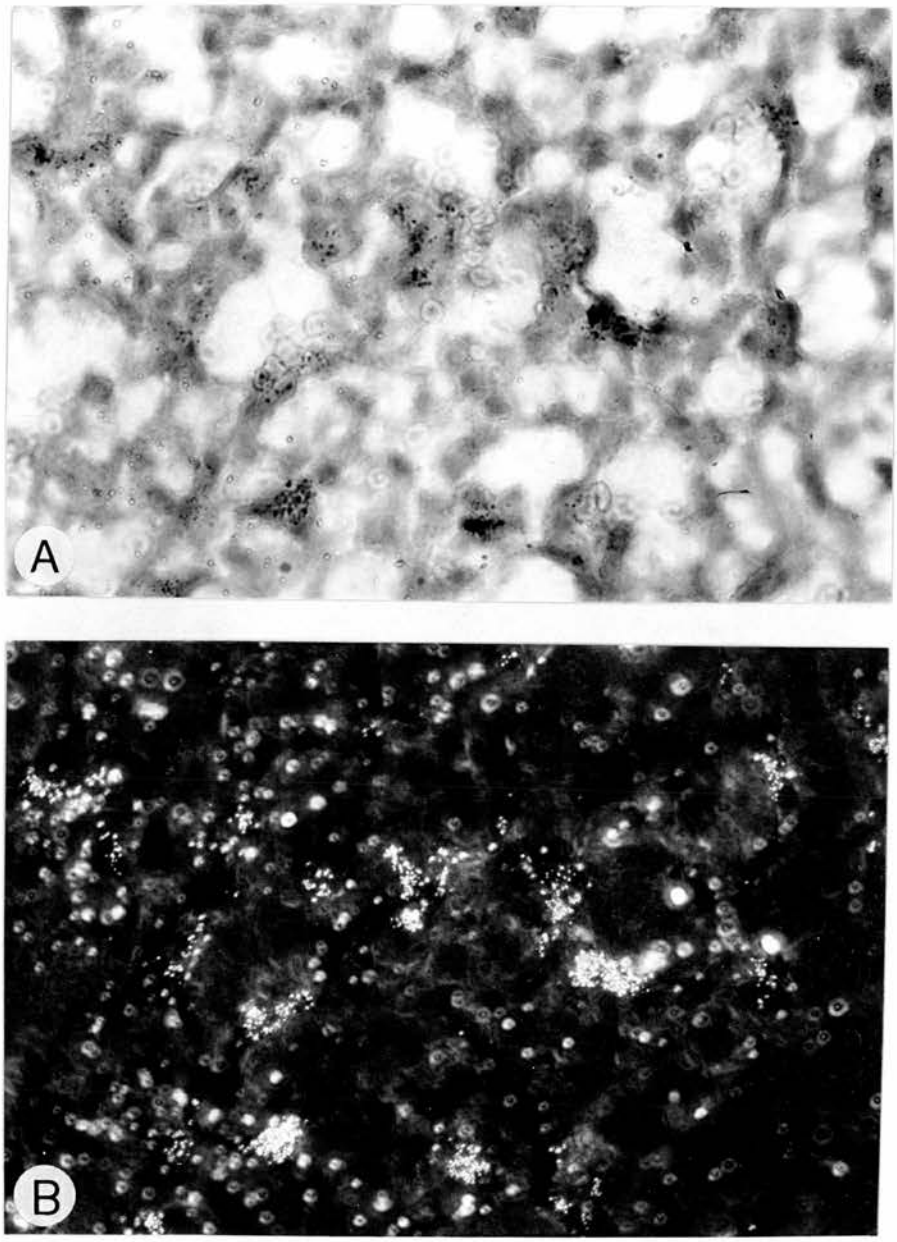

PLATE 3.1 Autoradiographic localization of ^{125}I-LHRH agonist binding by light (A) and dark (B) field illumination in rat pituitary cells from rats treated with ^{125}I-LHRH agonist. X 500.

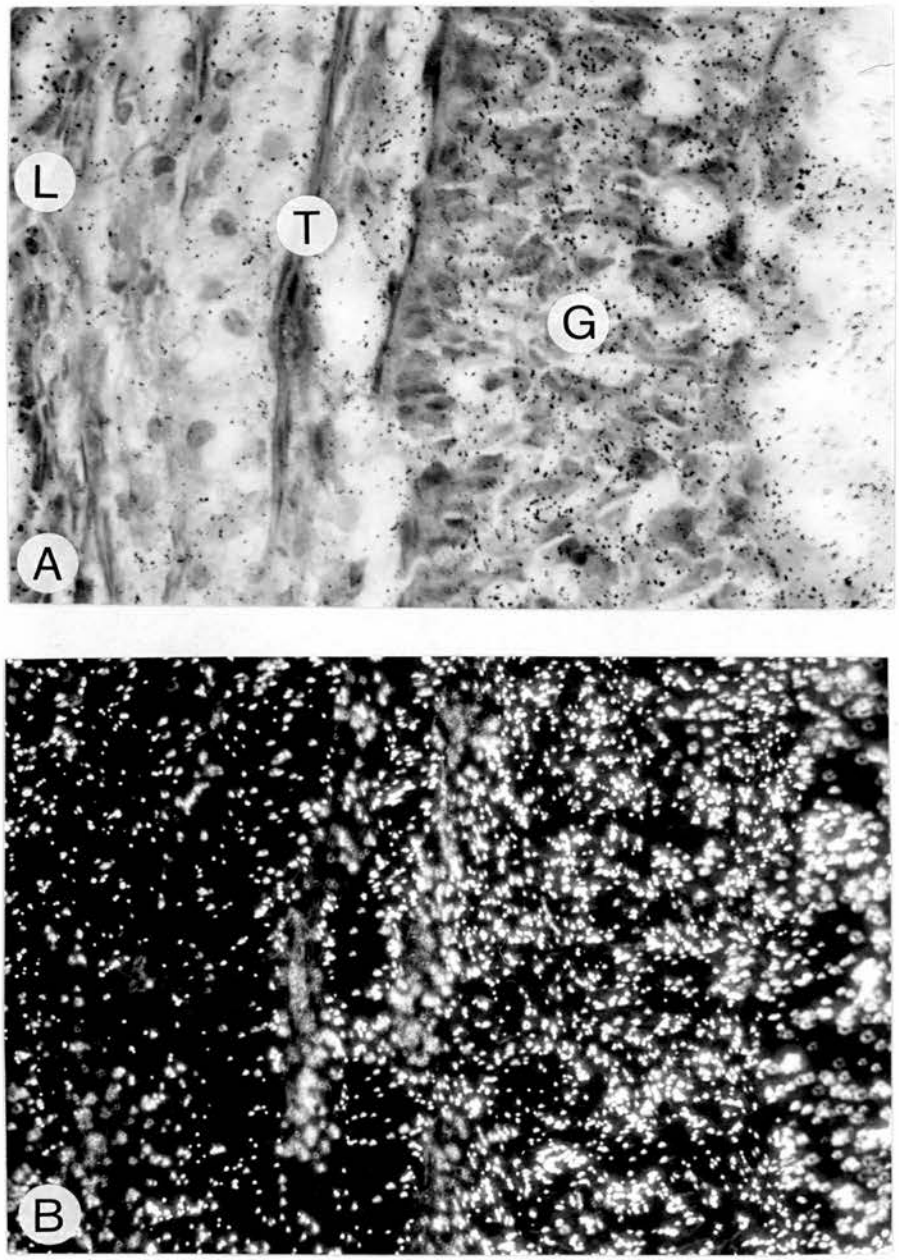

PLATE 3.2 Autoradiographic localization of ^{125}I-LHRH agonist binding by light (A) and dark (B) field illumination in rat ovarian tissue from rats treated with ^{125}I-LHRH agonist. Grains are shown to be located over granulosa (G), thecal (T) and luteal (L) tissue. X 500.

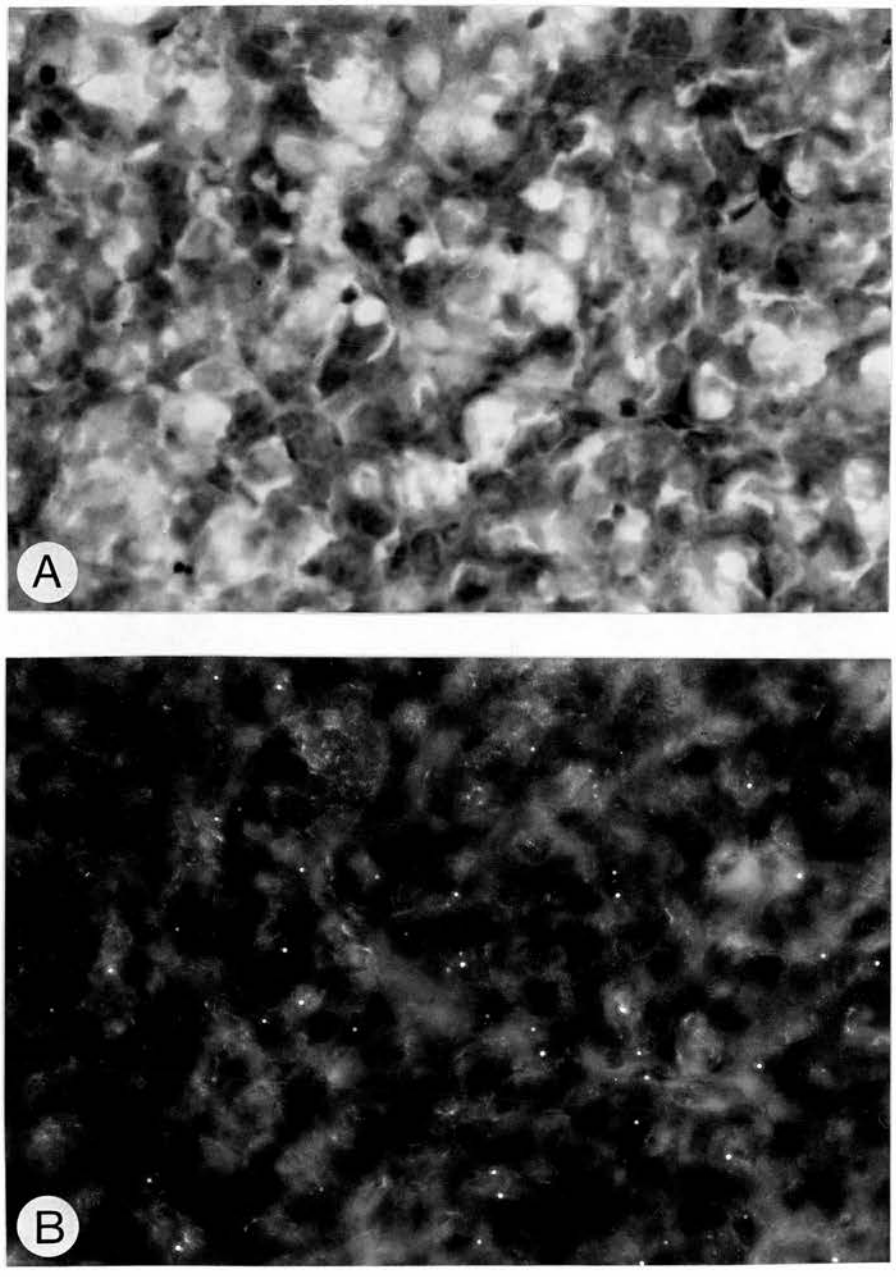

PLATE 3.3 Autoradiographic localization of ^{125}I-LHRH agonist binding by light (A) and dark (B) field illumination in control pituitary tissue from rats treated with an excess of cold LHRH agonist together with ^{125}I-LHRH agonist. X 500.

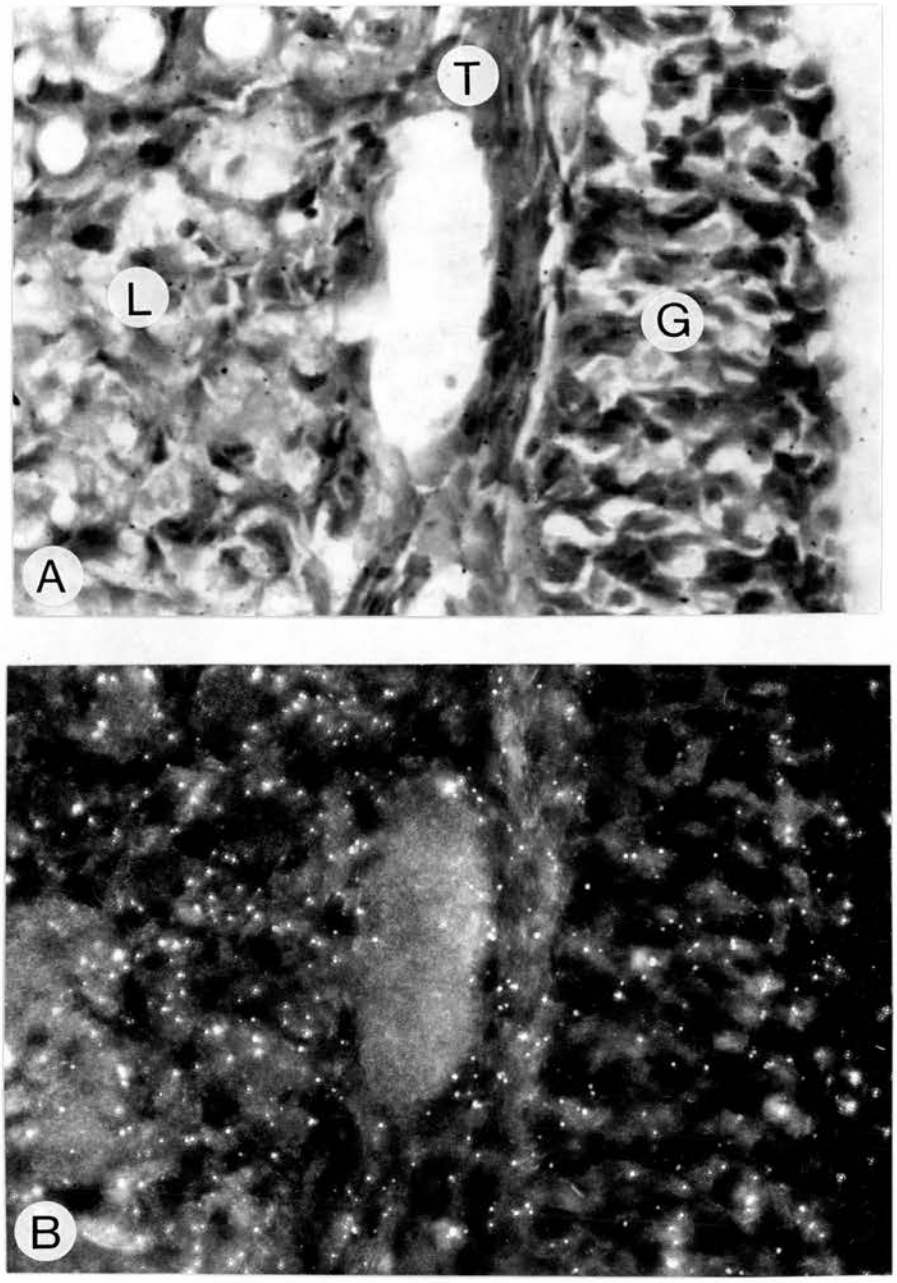

PLATE 3.4 Autoradiographic localization of ^{125}I-LHRH agonist binding by light (A) and dark (B) field illumination in control ovarian tissue from rats treated with an excess of cold LHRH agonist together with ^{125}I-LHRH agonist. X 500. Granulosa cells (G), thecal cells (T) and luteal cells (L).

examined microscopically at high power (x 1000) under oil-immersion and with bright-field and dark-ground illumination.

This experiment was performed in collaboration with Dr. R. Gosden.

3.2.5.3 Results

Both the ovaries and pituitary showed specific uptake of labelled ^{125}I-LHRH agonist. Uptake in the control animal, where binding had been displaced by excess of 'cold' hormone, was substantially lower.

Silver grains over pituitary tissue were unevenly distributed amongst cells of the adenohypophysis being primarily localized over small groups of cells (Plate 3.1). The grains over ovarian tissue were distributed throughout thecal, granulosa and luteal tissue (Plate 3.2). In both cases labelling in the control animals was markedly lower (Plates 3.3 & 3.4).

3.2.5.4 Discussion

These results emphasise the different binding patterns of LHRH in rat pituitary and ovarian tissue. In the pituitary, binding was located in discrete areas (presumably gonadotroph cells) whereas in the ovary, binding was more diffuse, being distributed throughout all cell types. Although these observations were based on only two animals, this pattern of ovarian binding has since been confirmed (Séguin et al., 1982; Pelletier et al., 1982a) and is in agreement with biochemical studies implicating direct effects of LHRH at multiple sites within the ovary (reviewed in Chapters 1 & 6).

That pituitary LHRH binding is confined exclusively to gonadotroph cells had been demonstrated by an elegant series of

experiments. The first morphological demonstration of binding employed ferritin-coupled LHRH (Hopkins & Gregory, 1977) followed by detailed autoradiographic localization of binding primarily over cells which stained immunochemically for LH (Duello & Nett, 1980; Pelletier et al., 1982b). In addition, enriched populations of gonadotroph cells prepared by velocity gradient sedimentation at unit gravity contained 71% of LHRH receptors (Naor et al., 1982).

Although fractionation of purified membrane preparations and subsequent receptor assay indicated that the majority of pituitary LHRH receptors were located in the plasma membrane fraction (Marian & Conn, 1983), pulse chase experiments have indicated that the LHRH-receptor complex is internalized. Several techniques have been utilized to confirm the intracellular fate of the LHRH-receptor. Autoradiography, (utilizing ^{125}I-LHRH agonist) followed by electron microscopy has revealed the presence of internalized receptor-bound hormone (Duello & Nett, 1980; Duello et al., 1983). Immunohistochemical techniques have localized LHRH inside gonadotroph cells (Bauer et al., 1981). One of the most exciting advances in this area however, has been the development of a rhodamine-conjugated LHRH derivative (Naor et al., 1981; Hazum, 1981a,c). This has enabled visualization of the ligand-receptor interaction by video-intensified fluorescence microscopy. Binding of rhodamine-labelled LHRH to dissociated rat pituitary cells was followed by aggregation along the cell surface (patching), association with coated pits (capping) and internalization into endocytic vesicles (Hazum et al., 1980, 1982a; Naor et al., 1981) according to the 'receptor mediated endocytosis' sequence reviewed by Goldstein et

al., (1979).

Interestingly however, although receptor internalization follows LHRH binding it is not a prerequisite for gonadotrophin release. Thus if internalization was prevented, e.g. using vinblastin, LHRH-stimulated LH release still occurred (Conn et al., 1981a; Conn & Hazum, 1981). The observation that a dimer molecule of LHRH antagonist was converted to a potent agonist upon addition of a cross reacting antibody (with resultant formation of a divalent antibody - dimer conjugate (Conn et al., 1982a,b)) led to the suggestion that receptor microaggregation in the lateral plane of the membrane was an important feature of LHRH action (Blum & Conn, 1982).

The physiological significance of internalized receptor complexes remains unknown, but is likely to be involved in receptor turnover (Marian & Conn, 1983) and degradation of LHRH (Duello et al., 1983).

Only limited data is available as yet on the fate of ovarian LHRH receptors in the different ovarian compartments. However, the same rhodamine-LHRH derivative has indicated that receptor-mediated-endocytosis occurred, with internalization of LHRH, in isolated granulosa cells (Hazum & Nimrod, 1982). Whether this is a prerequisite for biological action, or a means of receptor turnover, still remains to be established.

The techniques outlined above have dramatically increased our understanding of the histological location of LHRH binding and events subsequent to receptor activation. Knowledge gained so far has provided the basis for current theories on the mechanism of receptor

regulation (discussed in Chapter 9).

3.3 BIOCHEMICAL NATURE OF THE LHRH RECEPTOR

From the LHRH binding studies outlined earlier it seemed evident that gonadal and pituitary LHRH receptors were identical, apart from the lower receptor concentration in the former. A number of studies have attempted to characterize the biochemical nature of the pituitary receptor and it is interesting to review the data available and compare it with that on gonadal receptors.

The pituitary LHRH receptor is likely to be a glycoprotein with an important sialic acid moiety, since binding is abolished after treatment with neuraminidase, trypsin or chymotrypsin (Hazum, 1981c, 1982). A number of groups have attempted to isolate the receptor. For example, using the rhodamine LHRH derivative, photolabelling of the rat pituitary receptor (after sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)) was found to be associated with two protein bands; a major component of apparent molecular weight 60,000, and a minor component of 48,000 daltons (Hazum, 1981c). An identical estimated molecular weight of 60,000 was obtained by an independent group using receptor protein isolated from steer pituitaries. The isolated protein showed ^{125}I-LHRH binding characteristics similar to those of intact membranes (Zolman & Valenta, 1978). Recent studies have utilized a zwitterionic detergent, CHAPS (3[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate) to solubilize pituitary LHRH receptors. A large molecular weight complex with LHRH binding characteristics identical to those found in whole tissue has been isolated from bovine anterior pituitary tissue (Perrin *et al.*, 1983; Winiger *et al.*, 1983) with an

apparent molecular weight of 80,000 (Winiger et al., 1983). The isolated receptor complex was suggested to be composed of aggregated molecules, and no similar complex could be isolated from rat kidney tissue (Perrin et al., 1983).

Digestion of pituitary membrane phospholipids and phospholipases reduces LHRH binding (Hazum et al., 1982b). Since phospholipids are essential for maintaining structural integrity of the membrane, it is evident that the fully functional receptor is dependent on plasma membrane association.

Application of the photolabelling technique for isolating granulosa cell LHRH receptors led to the identification of two specific components of molecular weight 60,000 and 54,000 (Hazum & Nimrod, 1982), the former being identical to the estimate from pituitary tissue (see above, Hazum, 1981c). However, the 54,000 molecular weight component appears to be specific to the gonad, the pituitary minor component being estimated at 48,000 daltons. LHRH receptor sites at the two loci may therefore not be identical. This hypothesis has also been investigated in the testis. Comparison of the effects of enzyme treatment on LHRH agonist and antagonist binding to pituitary and Leydig cells, revealed differences. Whereas chymotrypsin and trypsin decreased binding in both tissues, neuraminidase was effective only in decreasing pituitary binding and actually increased Leydig cell binding (Millar et al., 1982). This suggests that a sialic acid residue is important for pituitary, but not Leydig cell, LHRH binding.

In conclusion, it appears that the binding characteristics as regards specificity and affinity, of LHRH receptors both in the

pituitary and gonad, are indistinguishable. The distribution of receptors differs however, being of greater concentration and on specific cell types within the pituitary, but being more diffuse in the ovary (Plates 3.1-3.4). Finally, the biochemical nature of one of the receptor subunits may differ between pituitary and gonad perhaps reflecting differences in the characterisics of the endogenous ligand.

CHAPTER 4

REGULATION OF PITUITARY LHRH RECEPTORS

CHAPTER 4

REGULATION OF PITUITARY LHRH RECEPTORS

4.1 INTRODUCTION

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4.4 LHRH RECEPTOR REGULATION IN THE FEMALE

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4.4.6 Summary of Results and Conclusions

4.1 INTRODUCTION

Regulation of the number and/or affinity of target cell receptors provides one mechanism by which responsiveness of a target cell to a particular ligand can be regulated. There are numerous examples of this in endocrinology (see Roth, 1979; Catt et al., 1979). Where receptor numbers are limiting, logically it would be expected that alterations in number would lead to changed magnitude of the biological response. However, since, for LHRH, occupancy of only 20% of receptors is required to elicit maximum biological response (Naor et al., 1980) it is evident that, as with gonadal LH receptors (Catt & Dufau, 1973) "spare receptors" are present. In the case of alterations in LH receptor concentrations, receptors have been shown to correlate with changes in target cell responsiveness (Harwood et al., 1978 & see Catt et al., 1979); likewise changes in the numbers of LHRH receptors could, theoretically, influence pituitary responsiveness.

The previous chapter described methods utilized to measure LHRH receptor concentrations and discussed the concept of receptor turnover providing a mechanism for regulating receptor concentration. This chapter is concerned with identification of some of the potential molecules regulating pituitary LHRH receptor numbers.

4.2 REVIEW - CORRELATION BETWEEN PITUITARY RESPONSIVENESS AND LHRH RECEPTORS

4.2.1 Sexual maturation

Pituitary LHRH receptors show increased concentration from

birth, reaching peak values at 30 days of age in males and 20 days in females, falling thereafter to adult levels at 80 and 40 days old respectively (Dalkin et al., 1981; Chan et al., 1981). These changes correlate well with the observed alterations in pituitary responsiveness to LHRH which is maximal at 30-40 days of age in males and 20 days in females (Debeljuk et al., 1972a,b; Ojeda et al., 1977). This change in receptors represents a change in concentration. Since pituitary protein content is increasing with age, expression of receptor numbers per gland shows a steady increase which is maintained from day 30 in both sexes (Chan et al., 1981). It is possible that this observation reflects alterations in the number of gonadotroph cells, which are known to change at this time (Denef et al., 1978), rather than receptor numbers per cell.

4.2.2 Adulthood

Pituitary protein content varies little during adult life so expression of receptor numbers per gland gives an adequate means of assessing concentration. Pituitary LHRH receptors do not vary during adulthood in male rats, and correspondingly there is no evidence for changes in pituitary responsiveness at this time. However in cycling female rats it is known that responsiveness shows marked changes (e.g. Aiyer et al., 1974a). Initial studies of pituitary receptors during the oestrous cycle indicated that maximum binding occurred at pro-oestrus (Park et al., 1976), the time of maximum responsiveness (Aiyer et al., 1974a). Although this study (Park et al., 1976) utilized ^{125}I-LHRH as ligand, the cyclic pattern of receptor numbers has been confirmed by numerous other studies utilizing

^{125}I-LHRH agonist (e.g. Savoy-Moore et al., 1980; Clayton et al., 1980). The characteristic binding pattern showing high levels at pro-oestrus and nadir at oestrus, was demonstrated in both isolated cell (Meidan & Koch, 1981) and tissue homogenates or membrane preparations (Clayton et al., 1979b; Savoy-Moore et al., 1980; Clayton et al., 1980). In addition the same binding changes have been found utilizing ^{125}I-LHRH antagonist (Meidan & Koch, 1981). No evidence has been found for changes in receptor affinity (e.g. Savoy-Moore et al., 1980 Marian et al., 1981; Meidan & Koch, 1981).

These observations were not confined solely to the rat. Similar cyclic changes in pituitary LHRH receptors were also noted in the hamster (Adams & Spies, 1981a). In addition, receptor numbers are elevated during oestrus in ewes (Crowder & Nett, 1982) and following oestrogen administration to ovariectomized Rhesus monkeys (Adams et al., 1981).

4.2.3 Pregnancy and Lactation

Lactation is associated with a decreased pituitary responsiveness to LHRH (Lu et al., 1976; Smith, 1978) and measurements of pituitary LHRH receptors have shown a marked reduction in numbers (but no affinity change) at this time in rats (Marian et al., 1981; Reeves et al., 1982). Pituitary receptor numbers were unchanged during pregnancy relative to oestrous levels in rats (Reeves et al., 1982). Pregnant beef heifers showed receptor numbers similar to those at oestrus (Schoenemann et al., 1982). Pseudopregnant rats had fewer LHRH receptors than weanling females

(Marian et al., 1981); however, since receptor numbers are highest at this time it is perhaps more meaningful to use adult cycling values as comparison. It is unlikely therefore that receptor numbers during pregnancy are significantly different from those of cycling rats.

4.2.4 Castration

Removal of the inhibitory feedback influence of gonadal steroids on gonadotrophin release is known to result both in marked increases in serum LH concentrations and in pituitary responsiveness to LHRH (O'Conner et al., 1980; Frager et al., 1980). Numbers of pituitary LHRH receptors are elevated following gonadectomy in both males and females (e.g. Clayton & Catt, 1981b). In male rats both the LH and receptor response is rapid, with increased receptors detectable 18-24 h after castration (Frager et al., 1981; Clayton & Catt, 1981b). However, in the female, both the gonadotrophin and receptor responses are slower than in the male, no change being seen until 3 days after ovariectomy (Frager et al., 1981; Clayton & Catt, 1981b). The reason for the sex difference is unclear, although adrenal steroids have been implicated in the case of the female (Clayton & Catt, 1981b). The important observation though is that in both cases elevated receptor numbers correlated well with elevated serum LH, whereas receptor affinity remained unchanged (Clayton & Catt, 1981b).

4.2.5 Assessment of pituitary receptors in anoestrous rats

Ageing is associated with changes in receptor numbers, but not affinity, for a variety of hormones in rats, mice, dogs and humans

(Roth, 1979). Since ageing rats show a tendency to enter a state of constant oestrus and loss of reproductive function (Watkins et al., 1975) it is possible that this change is associated with altered pituitary LHRH receptor numbers. Similar loss of reproductive function can be induced by maintaining female rats in constant light for periods of 2-3 months. Therefore in the experiment described receptor numbers were assessed after exposure to constant light.

Method: 10 regularly cycling female rats were kept in constant light (intensity 400 lux) for 10 weeks. These animals were then killed, together with 10 cycling rats of the same age, killed either at 10.00 h dioestrus or at noon pro-oestrus, using dry ice CO_2. Anterior pituitaries were removed and stored at -40°C prior to assaying LHRH binding as described in Chapter 3.

Results: All animals kept under constant light exhibited constant oestrous type vaginal smears of cornified cells. Ovarian histology indicated the presence of cystic follicles (Lawton & Schwartz, 1967; Reiter & Klein, 1971; see Chapter 5). Pituitary LHRH receptor numbers are shown in Fig. 4.1. Cycling rats showed the expected increased receptor numbers at pro-oestrus ($P < 0.05$). However receptor numbers from pituitaries from constant light rats were not significantly different from those of pro-oestrous cycling rats, although they were significantly raised above the low levels at dioestrus.

Discussion: The absence of changes in LHRH receptor numbers in the presence of marked alterations in reproductive function is surprising. However it is in agreement with observations that

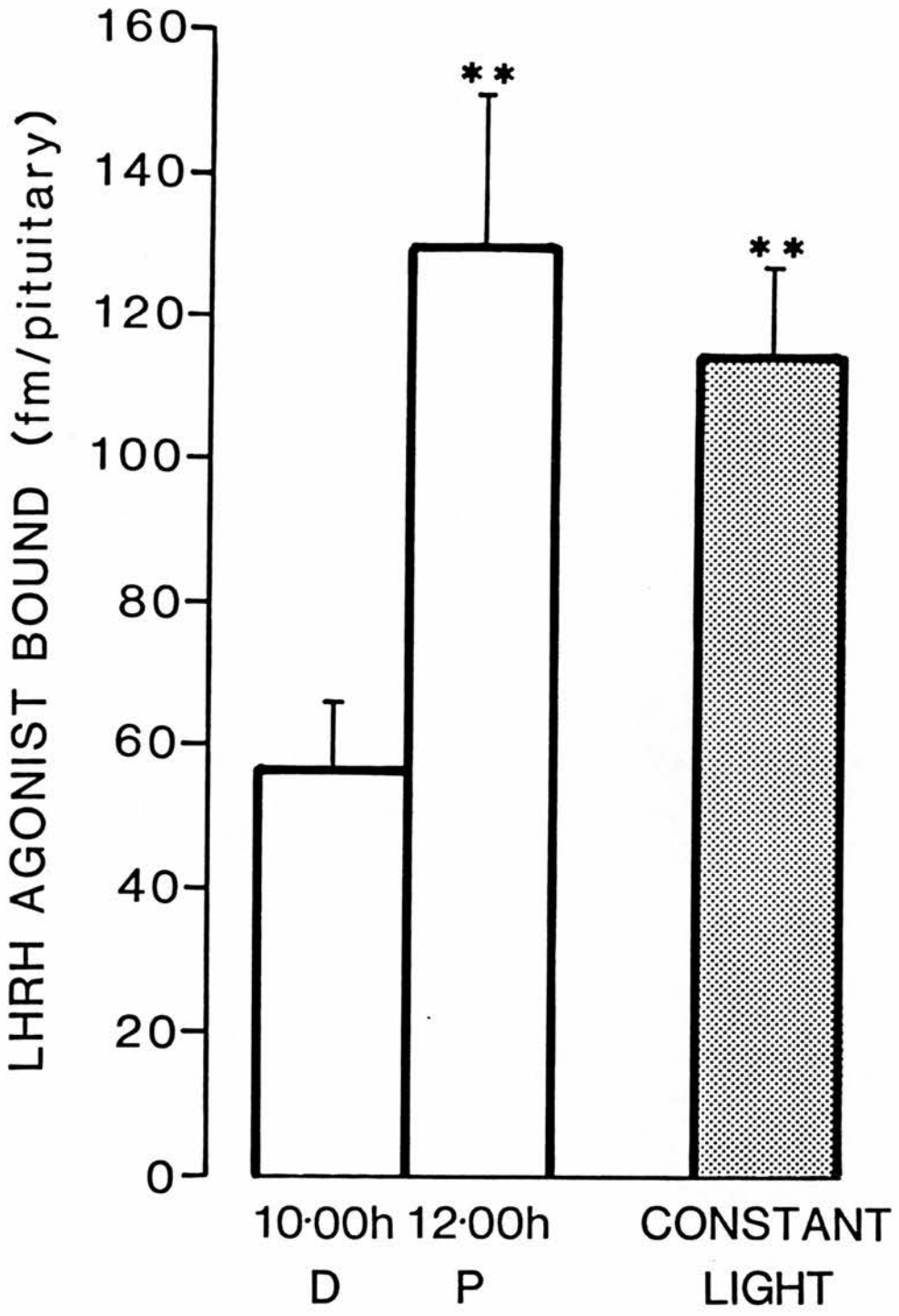

FIGURE 4.1 Pituitary LHRH receptors in control animals killed at 10.00 h dioestrus (D) or 12.00 h pro-oestrus (P) and in rats killed after exposure to constant light for 10 weeks.

Data represent Mean ± S.E.M. (10 constant light, 5 for each group in controls).

** $P < 0.01$ compared to D control value.

pituitary responsiveness to LHRH does not vary in rats exposed to constant light (Fink, 1975). Studies on pituitary receptor concentrations from aged or spontaneously acyclic rats were found to show decreased receptor numbers compared with immature rats (Marian et al., 1981). However, this comparison is misleading due to elevated receptor concentrations in prepubertal rats (see 4.1) and when compared with cyclic females, receptor numbers were not significantly reduced (Marian et al., 1981).

Conclusion: Pituitary LHRH receptor concentration was not altered from the cyclic range after exposure to constant light. It is therefore likely that pituitary responsiveness to LHRH at this time is regulated by other factors e.g. the rate or quantity of LHRH released.

4.3 REGULATION OF PITUITARY LHRH RECEPTORS IN THE MALE

4.3.1 Introduction, Aims, Models

Introduction

The fact that pituitary receptor numbers correlate well with changes in pituitary responsiveness to LHRH during sexual maturation (4.2.1), the oestrous cycle (4.2.2) and lactation (4.2.3), has led to the suggestion that the relationship is a causal one i.e. that changes in receptor numbers are responsible for the observed changes in pituitary responsiveness (Belchez et al., 1978). Since two categories of molecule are thought to regulate pituitary responsiveness, namely heterologous ligands such as gonadal steroids and LHRH itself (see Chapter 1), the mechanism of action may well involve changes in LHRH receptors. In order to minimize cyclic

variation in pituitary responses, studies were initiated in the male.

Aims

1. To investigate the ability of heterologous ligands (gonadal steroids and prolactin) to alter pituitary LHRH receptors.
2. To investigate the ability of LHRH to autoregulate its own receptors.

Experimental Models

Receptor changes were investigated after the following procedures had been utilized to disrupt the hypothalamic-pituitary-gonadal axis.

i) Specific removal of the negative feedback influence of testosterone by active immunization against testosterone (4.3.1.1.) or by castration (4.3.1.2.).

ii) Hyperprolactinaemia induced by transplantation of pituitaries under the kidney capsule (4.3.1.2.).

iii) Specific removal of endogenous LHRH (and subsequent inhibition of both gonadotrophin and steroid levels) following either active (4.3.2.1) or passive (4.3.2.2) immunization against LHRH.

4.3.2 Heterologous ligand regulation

The ability of molecules, other than the ligand responsible for binding to and initiating a response from a target cell, to influence receptor numbers for that ligand is not unusual in biological systems. In the case of the pituitary, responsiveness to thyrotropin releasing hormone (TRH) is known to be increased by oestradiol and decreased by prolactin (De Lēan et al., 1977b). These changes are

associated with increased and decreased pituitary TRH receptors respectively (De Léan et al., 1977a). Since pituitary responsiveness to LHRH is also regulated by heterologous molecules, it was decided to investigate the ability of these hormones to alter receptor numbers.

4.3.2.1 Active Immunization against Testosterone

Introduction: Since pituitary LHRH receptors are raised following the removal of gonadal steroids (see 4.2.4) it seemed reasonable to suggest that the negative feedback effects of steroids on gonadotrophin release could, in part at least, be due to a reduction in pituitary receptor numbers. In order to assess the inhibitory role of testosterone, the effects of specific immunization of testosterone on pituitary LHRH receptors was investigated in intact rats.

Method: 20 male Sprague Dawley rats (90 days of age) were actively immunized either against testosterone or against BSA in Freund's complete adjuvant (see chapter 2). Booster immunizations were given at 6 and 11 months using Freund's incomplete adjuvant. Rats were killed one month after the final booster using dry ice CO_2 and blood collected by decapitation, the serum being stored at -20°C prior to hormone assay and antibody titre determination. Anterior pituitary glands were used immediately for the binding assay as described in Chapter 3. Anti-testosterone antibody titre was determined and serum testosterone was measured after acidification of serum with an equal volume of 1 M HCl to release antibody bound steroid, as described in Chapter 2. Serum and pituitary content of

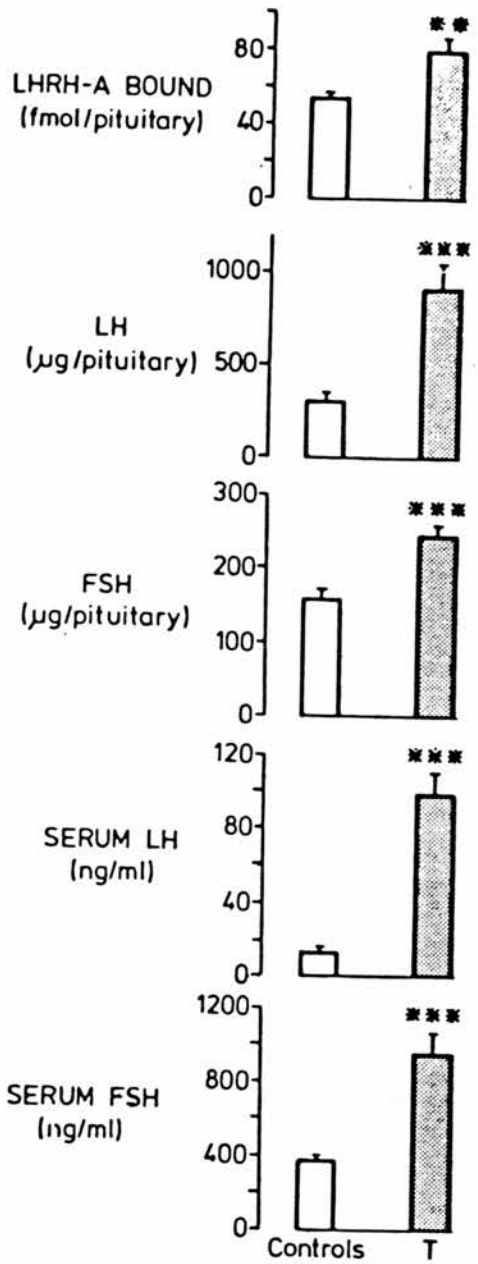

FIGURE 4.2 Effect of active immunization against testosterone (T) or HSA (control) on pituitary LHRH receptors, pituitary content and serum concentrations of LH and FSH in adult male rats.

Data are Mean ± S.E.M. (10 animals per group).

** $P < 0.05$; *** $P < 0.001$.

gonadotrophins were measured by radioimmunoassay (see Chapter 2).

Results: In testosterone-immunized rats the titre of antibody varied between animals from as little as 1:50 to a maximum of 1:4,000, with the majority of animals exhibiting high titre in the range 1:800 - 1:4,000. Despite this variation, the antibodies were clearly effective in all the testosterone-immunized animals as evidenced by the raised ($P < 0.001$) serum levels and pituitary contents of LH and FSH (Fig. 4.2). Serum concentrations of testosterone were markedly increased in the testosterone-immunized rats (controls 1.2 ± 0.2 ng/ml; immunized 61.7 ± 9.6 ng/ml; $P < 0.001$; Mean $\pm$ SEM). There was a significant increase ($P < 0.05$) in LHRH receptors in the testosterone-immunized animals (Fig. 4.2).

Discussion: The reduction in available testosterone induced by the circulating antibodies, led to an increased pituitary content and serum concentration of LH and FSH, and was associated with a significant increase in the number of pituitary LHRH receptors. Since the increases in both parameters were less pronounced than those following castration (see section 4.3.2.2 and Clayton & Catt, 1981b) it is possible that small amounts of non-antibody-bound testosterone are present in the immunized rat, whereas castration completely removes the source of testosterone. The data are consistent however with the concept of an inhibitory effect of testosterone on LHRH receptor numbers. This has also been demonstrated in intact rats by exogenous administration of steroid. Daily injection of testosterone (500 µg/day for 11 days) or implantation of testosterone capsules to intact rats led to a

reduction in the number of pituitary LHRH receptors (Naess et al., 1981; Heber et al., 1982).

The majority of studies confirming this concept have come from examination of the receptor changes following gonadectomy and steroid replacement therapy. In general, acute testosterone replacement (administered from the time of castration, or up to 6 days later) prevents the post-gonadectomy receptor rise in both males and females (Naess et al., 1981; Frager et al., 1981; Conne et al., 1982; Marchetti et al., 1982). Other steroids show similar inhibitory effects. Oestradiol and progesterone replacement has been reported to prevent the receptor rise (Naess et al., 1981; Frager et al., 1981; Conne et al., 1982). Administration of oestradiol 7 days after ovariectomy resulted in decreased receptor numbers within 3 h (Marian et al., 1981). It appears, however, that prolonged gonadectomy abolishes the receptor response to steroids and thus chronic administration (more than 7 days after gonadectomy) has no effect on receptors (Conne et al., 1982; Marchetti et al., 1982).

However, steroids do not always exert inhibitory actions on pituitary function. Savoy-Moore et al., (1980) noticed a correlation between the rising receptor numbers during dioestrus to pro-oestrus of the oestrous cycle, and serum oestradiol levels, and suggested that part of the positive feedback influence of oestradiol could be mediated by increased receptor numbers. Oestradiol has since been found to increase LHRH receptors in isolated pituitary cells (Meidan et al., 1981). In addition, since the administration of androgens resulted in decreased receptors in isolated cells (Giguère et al.,

1981), steroids may have a direct effect on the number of pituitary LHRH receptors. The pituitary possesses receptors for both oestradiol and testosterone (Notides, 1970; Naess et al., 1974) thus providing a means by which steroids could influence receptor turnover. Moreover, since recent evidence suggests that the isolated LHRH receptor is capable of specific binding of both oestradiol and testosterone (Zolman, 1983), a direct effect of steroids on LHRH receptors cannot be excluded.

Conclusion: Active immunization against testosterone was associated with a marked increase in pituitary LHRH receptors, consistent with a negative regulatory influence of testosterone.

4.3.2.2 Effect of hyperprolactinaemia on pituitary LHRH receptors

Since pituitary responsiveness and LHRH receptor numbers are reduced during lactation (see 4.2.3), a condition associated with raised prolactin (PRL) levels (McNeilly et al., 1978; McNeilly, 1980),it was decided to assess the ability of PRL to influence pituitary receptor numbers both in intact and castrate male rats. Serum prolactin levels were raised by placing pituitary grafts under the kidney capsule.

Method: Adult male (200-250 g) PVP rats were used both as donors and recipients of anterior pituitary glands which were allocated at random to treatment groups. Two pituitary glands were transplanted under the kidney capsule of 2 groups of 5 rats, while 2 groups of 5 control rats were sham operated. One group of control and one group of pituitary transplanted rats were castrated 7 weeks later. All animals were killed 14 weeks after transplant operation by the usual

procedure and serum analysed as described earlier. Anterior pituitaries (both the animals' own and the transplanted glands) were rapidly removed and used immediately for binding assay (Chapter 3).

This study was performed in collaboration with Dr. A.S. McNeilly who performed the transplant operations. PRL levels were assayed by Ms. J. Smith.

Results:

Intact Rats: Pituitary transplanted rats demonstrated significant increases in serum PRL (controls 39 $\pm$ 4 ng/ml; transplants 107 $\pm$ 13 ng/ml; means $\pm$ SEM; $P < 0.001$) indicating the effectiveness of pituitary transplantation in raising serum PRL. Serum gonadotrophins and pituitary gonadotrophin content were reduced in transplanted intact rats compared with controls (Fig. 4.3; $P < 0.001$). However, serum testosterone was unaltered (controls 3.5 $\pm$ 0.2 ng/ml; transplanted 3.1 $\pm$ 0.3 ng/ml; means $\pm$ SEM).

Pituitary LHRH receptors were significantly lowered in transplanted versus intact control rats ($P < 0.01$; Fig. 4.3).

Castrated Rats: Serum PRL levels were increased in transplanted rats (32 $\pm$ 4 ng/ml controls; 121 $\pm$ 12 ng/ml transplants means $\pm$ SEM; $P < 0.001$). Castration resulted in the expected raised pituitary content and serum LH and FSH levels compared with intact rats (Fig. 4.3; $P < 0.001$). Serum testosterone was < 0.1 ng/ml in castrate rats. Castration resulted in a marked rise in pituitary LHRH receptors compared with those of intact controls ($P < 0.001$). The presence of raised PRL in the transplanted rats had no significant effect on any of the parameters induced by castration.

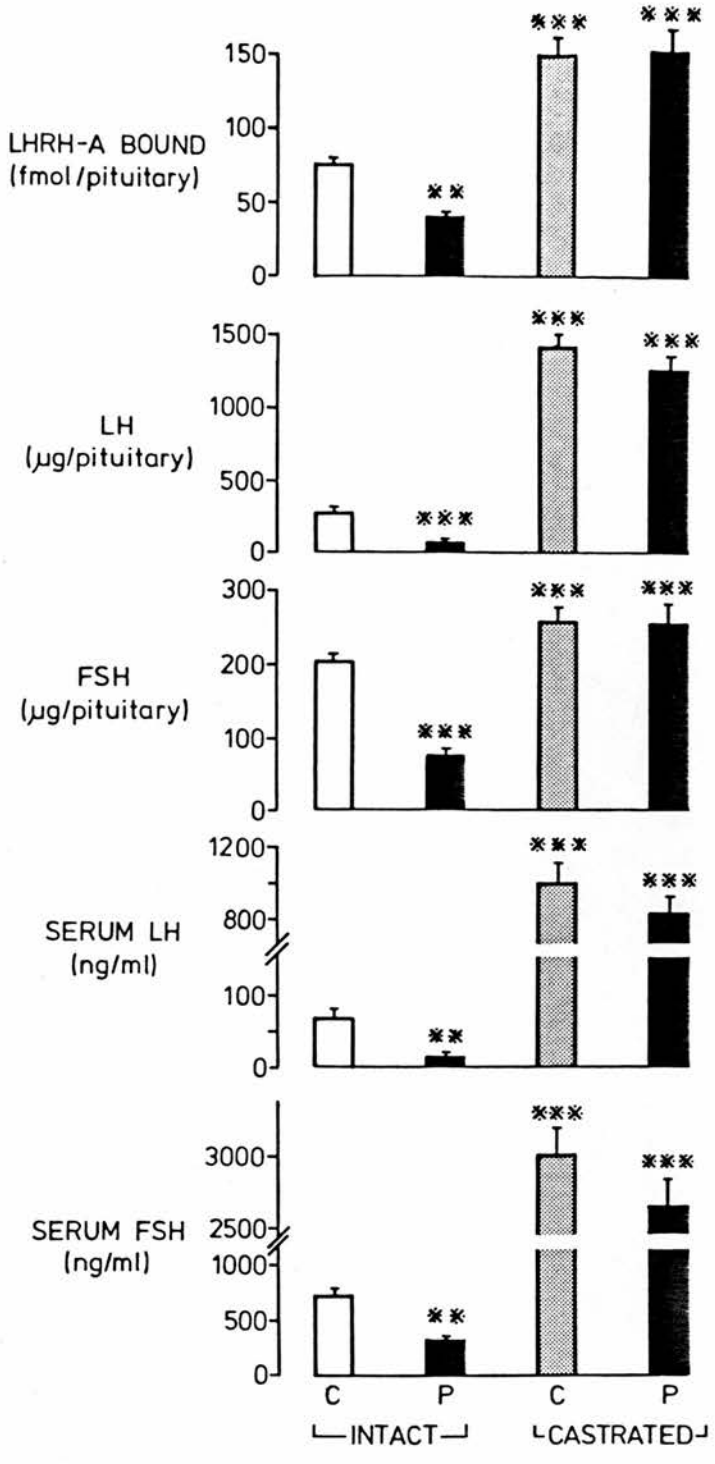

FIGURE 4.3 Effect of hyperprolactinaemia induced by pituitary transplants (P) on pituitary LHRH receptors, pituitary content and serum concentrations of LH and FSH in intact adult male control rats (C), in rats given a transplant of 2 pituitary glands 98 days previously (P) and left intact or castrated 49 days after receiving a pituitary transplant.

Data are Mean ± S.E.M. (5 animals per group).

** $P < 0.01$; *** $P < 0.001$ compared to intact control value.

TABLE 4.1

Pituitary LHRH receptors, gonadotrophin content and serum PRL and testosterone from glands transplanted under the kidney capsule

	Control	Castrate
LH (μg/pit)	7 ± 1	30 ± 14
FSH (μg/pit)	13 ± 1	7 ± 2
Serum PRL ng/ml	107 ± 13	121 ± 12
Serum Testosterone ng/ml	3.1 ± 0.3	$\lesssim$0.1
$LHRH_A$bound fmol/pit.	25 ± 3.9	24 ± 4.2

Transplanted Pituitaries: Results obtained from analysis of the LHRH receptor concentration and gonadotrophin content of the original two anterior pituitaries recovered from the kidney capsule, are shown in Table 4.1. Although the tissue had an atrophied appearance and decreased gonadotrophin content it still possessed LHRH receptors. In addition, there was no difference in receptor numbers from transplants in intact versus castrate rats.

Discussion: Previous work has shown that transplantation of pituitaries under the kidney capsule results in reduced serum and pituitary content of gonadotrophins, while leaving serum testosterone levels unaffected (Bartke et al., 1977; McNeilly et al., 1978). It is also known that, as in the lactating female, pituitary responsiveness to LHRH is reduced during hyperprolactinaemia (e.g. Winters & Loriaux, 1978). This decreased responsiveness may be due to decreased LHRH receptors as indicated by the above data. Since testosterone levels remained unchanged, the effects of hyperprolactinaemia on receptors in intact males are presumably mediated via PRL-induced changes alone. These data have been confirmed by Marchetti et al., (1982) who showed that injection of PRL, or raising endogenous levels by treatment of immature females for 14 days with sulpuride, resulted in decreased pituitary LHRH receptor content. These changes could be reversed by a dopaminergic agonist (Marchetti et al., 1982).

Short-term hyperprolactinaemia (3 weeks) had no effect on pituitary LHRH receptors in intact rats (Clayton & Bailey, 1982). However, prolonged hyperprolactinaemia clearly reduced receptor

numbers (Fig. 4.3). The importance of distinguishing between acute and chronic effects is further highlighted by the effect of hyperprolactinaemia on the receptor response to castration. Whereas the short-term exposure (7 days) to high PRL reduced the receptor increase (Clayton & Bailey, 1982; Marchetti & Labrie, 1982), prolonged hyperprolactinaemia had no effect (Fig. 4.3). This latter observation correlates with the finding that the long-term gonadotrophin response to castration is unaffected by pituitary transplants (McNeilly et al., 1980).

Since prolonged hyperprolactinaemia reduced LHRH receptors in intact but not castrate rats (Fig. 4.3) it could be suggested that the presence of testosterone is required for the long-term effects of prolactin on receptor numbers. This conclusion is supported by the observation that the decrease in post-ovariectomy response to short-term hyperprolactinaemia was enhanced by oestradiol (Marchetti & Labrie, 1982).

It is interesting to note that pituitary tissue transplanted under the kidney capsule, and thus deprived of LHRH, still possessed receptors, albeit at low levels indicating that exposure to the endogenous ligand is not a prerequisite for receptor expression. In addition, since transplant receptor numbers were not significantly different in intact and castrate rats, it could be suggested that steroid-mediated inhibition of receptor numbers does not occur in the absence of endogenous LHRH.

Conclusion: Hyperprolactinaemia was associated with a decrease in pituitary LHRH receptors in intact rats, but not in castrate rats,

indicating that gonadal steroids may play a contributory role in receptor regulation under these conditions.

4.3.2.3 Inhibin and Opioids

Inhibin, a non-steroidal gonadal peptide is believed to regulate FSH release from the pituitary by influencing the responsiveness of the pituitary to LHRH (e.g. Bramble et al., 1975). That this may be brought about by receptor changes was suggested by the demonstration that daily subcutaneous injection of adult male rats with an inhibin extract, resulted in decreased LHRH receptor numbers (Sheth et al., 1982). However, the physiological relevance of this observation remains to be determined.

Opioids block the gonadotrophin surge and ovulation in rats (e.g. Pang et al., 1977; Köves et al., 1981). Morphine and naloxone administration has been shown to result in changes in pituitary LHRH receptor concentration (Barkan et al., 1983a) thus implicating a role for endogenous opioids in the regulation of pituitary LHRH receptors. Whether this effect is mediated by a direct action on the pituitary or via the hypothalamus (Ching, 1983) is unknown.

4.3.3 LHRH receptor Autoregulation

4.3.3.1 Introduction

A number of receptor systems appear to be regulated by the ligand itself - so termed autoregulation. This phenomenon was first observed when it was noticed that binding of labelled insulin to cultured lymphocytes decreased after exposure to high insulin levels (Gavin et al., 1974). This effect has been termed "down-regulation" and has been reported following exposure of tissues

to high doses of other hormones e.g. LH (Conti et al., 1976; Sharpe, 1976), growth hormone (Lesniak & Roth, 1976), TRH (Hinkle & Tashjian, 1975) and EGF (Heldin et al., 1979). However receptor autoregulation is not invariably inhibitory. For example, administration of lower doses of angiotensin II resulted in an increase in glomerula cell receptors (Hauger et al., 1978; Aguilera et al., 1978) i.e., "up-regulation".

A realization that LHRH autoregulation was likely to be involved in the determination of LHRH receptor numbers came from the observation that a number of endocrine states associated with changes in pituitary LHRH receptors are also correlated with altered endogenous LHRH release. Thus, immunoreactive hypothalamic LHRH increases from birth to day 21 of life (Araki et al., 1975), correlating with receptor changes (although whether this is released LHRH or not is unclear). LHRH is thought to be reduced during the post-partum period (Lu et al., 1976) and LH pulse frequency is reduced during lactation and hyperprolactinaemia, suggesting decreased LHRH release (Bohnet et al., 1975). Similarly, after castration, hypothalamic LHRH stores are depleted (Gross, 1980) and this process can be reversed by treatment with testosterone or oestradiol benzoate (Gross, 1980). Depleted stores of LHRH implied that portal blood LHRH levels were increased and several studies have indicated that this is indeed the case after castration (e.g. Neill et al., 1977; Sherwood et al., 1980).

Due to the difficulties in sampling hypophysial portal blood

it has not been possible to correlate directly changes in LHRH release with changes in receptor numbers. However, the fact that steroids have been shown to influence LHRH release (e.g. Sarkar & Fink, 1979; Fink, 1979; Gross, 1980) renders it possible that the receptor changes discussed under "heterologous regulation" are in fact mediated, not by direct effects of steroids LH or PRL on LHRH receptors, but rather by altering LHRH release and subsequent autoregulation.

LHRH autoregulation has the potential therefore to be the primary mechanism responsible for changes in LHRH receptors. The following experiments set out to investigate this possibility using techniques to immunoneutralize endogenous LHRH levels.

4.3.3.2 Active immunization against LHRH in the intact male

In order to assess the importance of LHRH in the regulation of its receptors, endogenous LHRH was immunoneutralized by active immunization.

Method: 10 adult (90 day old) male Sprague Dawley rats were immunized, five against BSA and five against LHRH (see Chapter 2). Booster immunizations were given at 6 and 11 months using Freund's incomplete adjuvant. One month after the final booster the animals were killed and blood and pituitaries collected as previously described.

Results: LHRH antibody titre varied from 1:4,000 - 1:25,000 and was sufficient to reduce both serum levels and pituitary content of LH and FSH in all rats (Fig. 4.4). Serum testosterone levels were non-detectable ($\lesssim$ 0.1 ng/ml). None of the antibodies cross-reacted

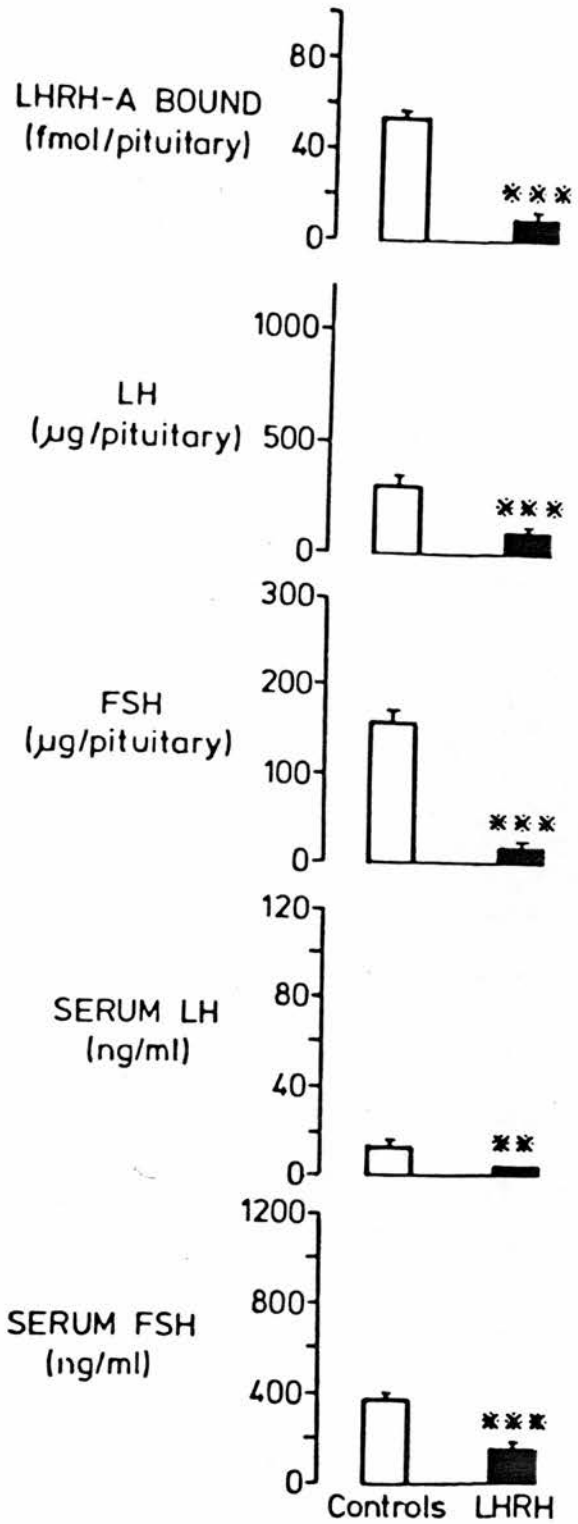

FIGURE 4.4 Effect of active immunization against LHRH (■) or HSA (□) on pituitary LHRH receptors, serum concentrations and pituitary content of LH and FSH in adult male rats.

Data are Mean ± S.E.M. (10 rats in control group, 5 LHRH immunized).

** $P < 0.05$; *** $P < 0.001$.

significantly with LHRH agonist, so were unlikely to interfere with the receptor binding assay.

Pituitary LHRH receptors were markedly reduced in LHRH-immunized rats ($P < 0.001$; Fig. 4.4) compared with intact controls.

Discussion: It can be assumed that active immunization against LHRH results in virtually complete inactivation of endogenous LHRH. Since receptor numbers were dramatically reduced after active immunization, this data provides strong evidence that LHRH autoregulation is involved in the maintenance of its receptors. In addition it is interesting to note that receptor numbers were not abolished after immunization, but reduced to 30% of normal.

4.3.3.3 Passive Immunization against LHRH

In order to assess the effects of short-term removal of endogenous LHRH on receptor numbers, the receptor changes following passive immunization were investigated. The LHRH antiserum used was raised in a ewe (No. 94) by immunization against LHRH conjugated to human serum albumin by carbodiimide. Specificity was directed towards the C-terminal end of the LHRH molecule (Ellis et al., 1983).

Method: 88 adult male Sprague Dawley rats were injected intravenously (under ether anaesthesia) with either 1 ml LHRH antiserum (No. 94) or with control BSA antiserum. Groups of 16 rats, 8 treated and 8 control, were killed 1, 6 or 24 h after antiserum injection.

The remaining animals were killed (5 control, 5 treated) 3, 7, 14 or 21 days after antiserum injection to assess receptor recovery.

Animals were killed and blood and anterior pituitaries collected

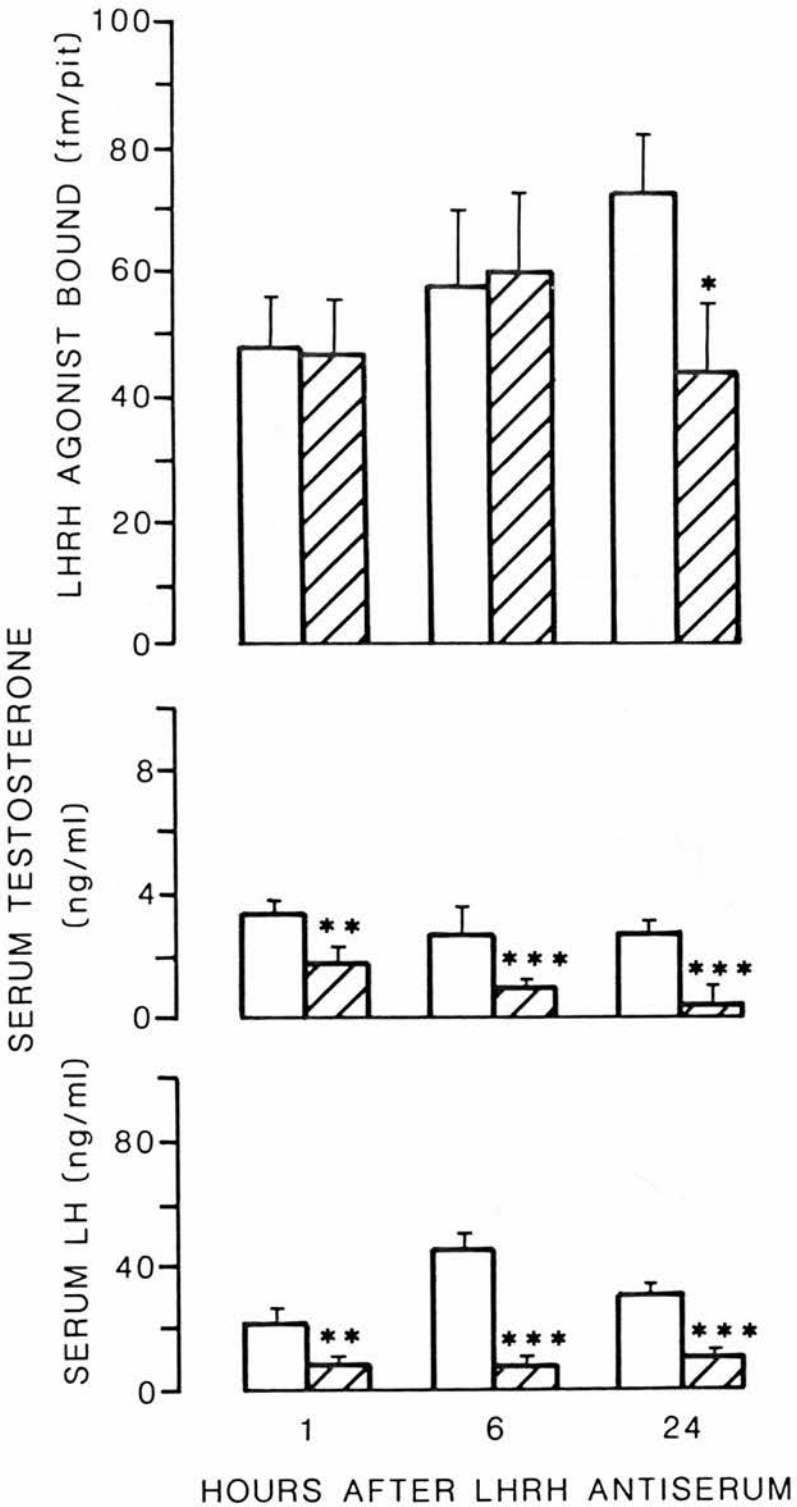

FIGURE 4.5 Pituitary LHRH receptors, serum testosterone and LH concentrations 1, 6 and 24 h after administration of either LHRH antiserum (▨) or control serum (□) to adult male rats.

Data are Mean ± S.E.M. (8 animals per group).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for LHRH antiserum treated compared to control values at each time point.

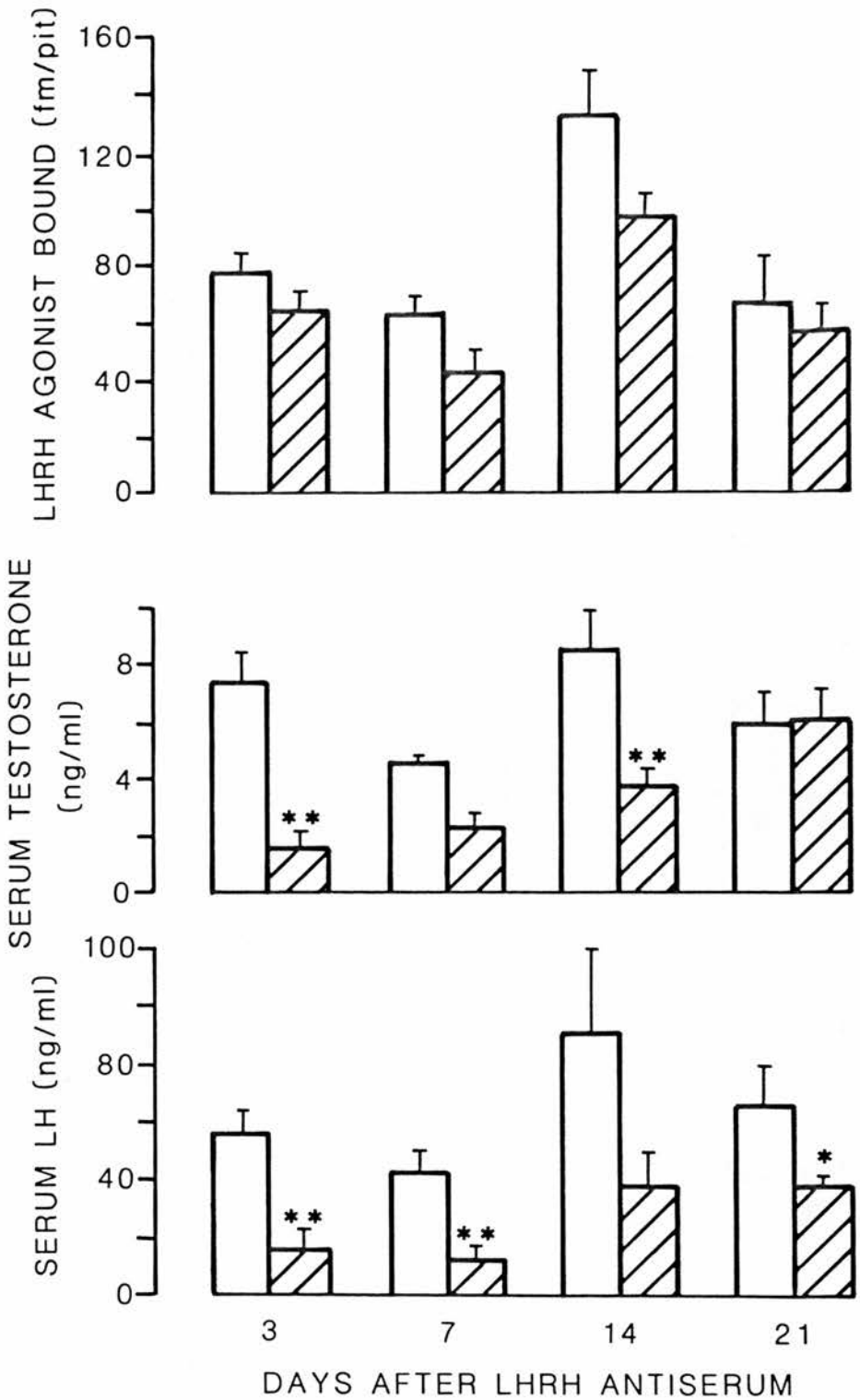

FIGURE 4.6 Pituitary LHRH receptors, serum testosterone and LH concentrations 3, 7, 14 and 21 days after administration of either LHRH antiserum (▨) or control serum (□) to adult male rats.

Data are Mean ± S.E.M. (8 rats per group).

* $P < 0.05$; ** $P < 0.01$ for LHRH antiserum treated compared to control values at each time point.

as described previously.

Results: Results are presented in Figs. 4.5 and 4.6. LHRH antiserum resulted in an immediate reduction in serum LH and testosterone levels ($P < 0.001$ after 1 h). Serum testosterone remained suppressed at 1, 3 and 14 days, returning to control values after 21 days. Serum LH levels were suppressed throughout the 21 day period although the low value on day 14 was not statistically significantly different from controls.

Pituitary LHRH receptors were significantly reduced only 24 h after antiserum injection ($P < 0.05$) but not after 1 or 6 h. Receptor numbers had returned to control levels 3 days after treatment.

Discussion: Antiserum administration clearly resulted in immediate immunoneutralization of endogenous LHRH as seen by the precipitous and maintained decreases in serum LH and testosterone levels (Figs. 4.5 & 4.6). This phenomenon has been noted following LHRH antiserum administration to rams (Lincoln & Fraser, 1979), ewes (Fraser & McNeilly, 1983) and castrate rats (Ellis et al., 1983) and emphasises the essential role of LHRH in the control of pituitary (and hence gonadal) function.

Several interesting observations can be made from the receptor data. Firstly, receptor changes are not correlated temporally with those of pituitary function. Thus the LHRH receptor decrease occured after the decrease in serum LH and testosterone (Fig. 4.5), suggesting that a prolonged period of reduced LHRH is necessary before receptor numbers are decreased. In addition, receptor recovery preceded that of serum LH and testosterone (Fig. 4.6).

Secondly, the fact that receptor numbers are unchanged 1 h and 6 h after antiserum injection, despite the obvious presence of high antibody titre as seen by the fall in LH, suggests that the antiserum does not interfere with the LHRH receptor assay. Thirdly, since receptor recovery preceded that of pituitary gonadotrophin release, it could be suggested that only small quantities of LHRH are required to initiate and maintain LHRH receptors. Thus, as LHRH antibody titre declines, sufficient LHRH is available to induce receptor "upregulation" but not LH release.

Conclusions:

1) Data from this and the previous experiment confirm that LHRH receptors are under positive autoregulatory control.
2) Receptor autoregulation exhibits a different time course from LH release and receptor number is therefore not invariably correlated with pituitary responsiveness in the intact animal.
3) Passive immunoneutralization of LHRH provides a sensitive method for the investigation of receptor autoregulation in the intact animal, and the antibody used is unlikely to interfere in the receptor assay.

4.3.3.4 Autoregulation in the Male - General Discussion

In addition to the above data utilizing active and passive immunoneutralization of LHRH, the concept of a positive autoregulatory influence of LHRH on its receptors has been corroborated by studies in castrate males. That the post-castration increase in receptors is due to LHRH autoregulation, has been convincingly demonstrated. Thus, administration of LHRH antiserum

identical to that used in the present study, to acutely or chronically castrated rats, abolishes both the post-castration receptor increase and the elevated serum LH (Clayton et al., 1982b), receptor levels being lowered to values below those detected in intact control rats (Clayton et al., 1982b). Similarly, prevention of LHRH interaction with its receptors by infusion of LHRH antagonist also prevented or reduced the post-castration receptor response (Clayton et al., 1982a) confirming that it is the specific interaction of LHRH with its receptor, rather than receptor occupancy per se, which is responsible for the observed changes.

Further confirmation of the role of LHRH "up-regulation" was demonstrated by removal of endogenous LHRH via lesioning the medial basal hypothalamus (MBH). This resulted in an abolition of the post-castration receptor increase (Clayton et al., 1982a; Pieper et al., 1982). Receptors could be elevated to post-castration levels by administration of LHRH or LHRH agonist, to reverse the effects of MBH lesions or antibody treatment (Pieper et al., 1982; Clayton et al., 1982a). It appears, therefore, likely that the raised LHRH receptors following castration are due to raised endogenous LHRH which both initiates and maintains the increase.

LHRH administration concurrently with castration resulted in a small decrease in receptors seen on day 7 (Frager et al., 1981). Although the significance of this observation was not noted, in another study LHRH injections from day 1 - 11 of castration reduced binding (Naess et al., 1981). It could be suggested therefore that further increases of LHRH above the post-castration level can reach

the threshold level required for down-regulation of LHRH receptors (Naess et al., 1981).

In intact rats, the effects of exogenous administration of LHRH on pituitary receptors are complex, depending on both the dose and duration of exposure to LHRH. The majority of reports indicate that acute exposure (4 - 11 days) to either LHRH (Naess et al., 1981) or LHRH agonist (Marshall et al., 1980; Heber et al., 1980; Frager et al., 1981; Marchetti et al., 1982; Clayton, 1982) resulted in increased receptor numbers, thus confirming the up-regulation seen after LHRH replacement in lesioned or antibody-treated castrate rats (Pieper et al., 1982; Clayton et al., 1982a). Attempts at achieving down-regulation in the intact rat using high doses of LHRH or LHRH agonist have either resulted in no change in receptor concentration (e.g. Heber et al., 1982) or a decrease (Clayton, 1982). The duration of exposure was also important; thus administration of 200 ng LHRH agonist/day for 20 days increased receptors, but for 40 days or 60 days had no effect (Heber et al., 1982).

It is interesting to note that depriving the pituitary gonadotroph of endogenous LHRH stimulation either by MBH lesions, LHRH immunization or pituitary transplantation under the kidney capsule, did not result in total abolition of LHRH receptor concentration. The continued expression of receptors, albeit at reduced levels, in the absence of ligand, suggests that a basal or pre-programmed receptor level exists which is independent of autoregulation. This hypothesis has recently been corroborated by the demonstration of specific LHRH receptors in the pituitary of the

hypogonadal mouse (Young et al., 1983). Receptor numbers were similar to those found in immunized or lesioned rats i.e. indicative of an absence of LHRH. A basal level of receptors therefore appears to be present irrespective of pituitary exposure to LHRH. It could be suggested either that heterologous ligands maintain these basal receptor numbers or more likely, that they are independent of regulation.

4.3.4 LHRH Receptor Regulation in the Male - Summary

The experimental results obtained from studies in the male are summarized in Table 4.2. The results show:-

1) Active immunization against testosterone resulted in similar changes to those seen following castration. LHRH receptors were increased.

2) Hyperprolactinaemia was associated with a decrease in receptors in intact rats, but not in castrate rats.

3) Chronic active immunization against LHRH markedly reduced receptor concentrations.

4) Passive immunoneutralization against LHRH was associated with a decrease in LHRH receptors.

Conclusions:-

1) Gonadal steroids and PRL exert negative regulatory influences on LHRH receptor numbers.

2) LHRH autoregulation is primarily up-regulation. Receptor levels correlate with known or assumed changes in endogenous LHRH levels.

TABLE 4.2

Summary of data obtained from receptor studies in male rats.

Arrows indicate the influence of the treatment regime on a number of parameters pertinant to receptor regulation, showing the direction of changes relative to intact control rats.

Treatment	Endogenous LHRH	Pituitary LHRH Receptors	Serum LH	Gonadal Steroids
Active Immunization against testosterone	? ↑	↑	↑	↓↓
Castration	? ↑	↑↑	↑↑	Non detectable
Castration + hyper PRL	? ↑	↑↑	↑↑	Non detectable
Intact + hyper PRL	? ↓	↓	↓	No change
Active Immunization against LHRH	↓↓	↓↓	↓↓	↓↓
Passive Immunization against LHRH	↓	↓	↓	↓

4.4 REGULATION OF LHRH RECEPTORS IN THE FEMALE

4.4.1 Introduction, Aims, Models

Introduction: Pituitary LHRH receptors and pituitary responsiveness to LHRH vary during the oestrous cycle (4.2.2). From the results of studies in the male it was evident that both heterologous factors (e.g. gonadal steroids) and LHRH itself were capable of regulating LHRH receptor concentration. In contrast, chronic exposure to LHRH agonist was known to result in marked antifertility effects (Chapter 1.4.2) and this had been suggested to be due, in part, to down-regulation of pituitary LHRH receptors (Chapter 5.1).

Aims:

1) To assess whether antifertility effects induced by exposure to extremes of LHRH could be explained by decreased LHRH receptor concentrations.

2) To assess the relative importance of gonadal steroids, LH and LHRH in controlling receptor concentrations in the cycling female.

3) In particular to assess the role of receptor autoregulation during the oestrous cycle.

Experimental models: Pituitary LHRH receptor concentrations were assessed following disruption of the hypothalamic-pituitary-gonadal axis by the following procedures:-

i) Chronic hyperstimulation with LHRH agonist or chronic hypostimulation following LHRH antiserum treatment (4.4.2).

ii) Chronic neutralization of LH or LHRH by active immunization (4.4.3).

iii) Passive immunoneutralization of LH or LHRH at specific times during the oestrous cycle (4.4.4).

4.4.2 Effect of extremes of exposure to LHRH in the intact adult female rat

Introduction: In order to assess the effects of extremes of exposure to LHRH on the pituitary-ovarian axis, the effects of hyperstimulation of pituitary function via daily injections of various doses of LHRH agonist or hypostimulation following passive immunoneutralization of endogenous LHRH were assessed. It was hoped that analysis of pituitary LHRH receptors would reveal whether the expected antifertility effects produced by these means were due to decreased receptor numbers.

Method: Regularly cycling adult female Sprague Dawley rats (60-80 days old) were used and daily vaginal smears taken to assess vaginal cytology both before and during treatment.

Animals were divided into 6 groups of 8. Treatment commenced at random stages of the cycle. Rats in groups 1-3 were injected subcutaneously at 09.00 h daily for 3 weeks with 50, 500 or 5000 ng LHRH agonist dissolved in 1% gelatin and 0.9% (w/v) NaCl. Rats in Group 4 were injected with 0.5 ml of antiserum to LHRH (No. 94) once every 3 days. The first antiserum injection was given intravenously and the remainder subcutaneously. Control rats received either vehicle alone or antiserum to human serum albumin (HSA) and were killed on either day one of dioestrus (Group 6) or on pro-oestrus

TABLE 4.3

Effect of treatment with LHRH agonist or LHRH antiserum for 3 weeks on serum LH, FSH, progesterone and oestradiol concentrations, pituitary content of LH and FSH and uterine and ovarian weight.

Group	Treatment	Serum FSH (ng/ml)	Serum LH (ng/ml)	Pituitary FSH (μg)	Pituitary LH (μg)	Serum Progesterone (ng/ml)	Serum Oestradiol (pg/ml)	Paired ovarian wt (mg)	Uterine wt (mg)
1	50ng agonist	293± 25[b]	145±35	15± 2	176±12	14±2[c]	34± 4	128±11[a]	328±26
2	500ng agonist	592±160[a]	123±35	16± 1	182±21	16±1[c]	27± 4[a]	140± 8[c]	203±13[b]
3	5000ng agonist	911±134[c]	125±30	19± 1	148±16[a]	9±1[c]	29± 3[a]	92± 9	143± 2[c]
4	LHRH antibody	204± 20	56± 8[b]	21± 2	232±26	19±4[c]	48± 8	52± 4[c]	353±34
5	Control (Pro-oestrus)	143± 9[c]	57± 7[b]	19± 1	218±10	14±5[a]	107±10[c]	90±12	621±58[c]
6	Control (Dioestrus)	201± 7	111±17	19±13	205±21	28±2	43± 5	90± 4	313±23

Values are mean ± S.E.M. for 8 animals/group

Values significantly different from those of Group 6 rats: [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$ (t-test).

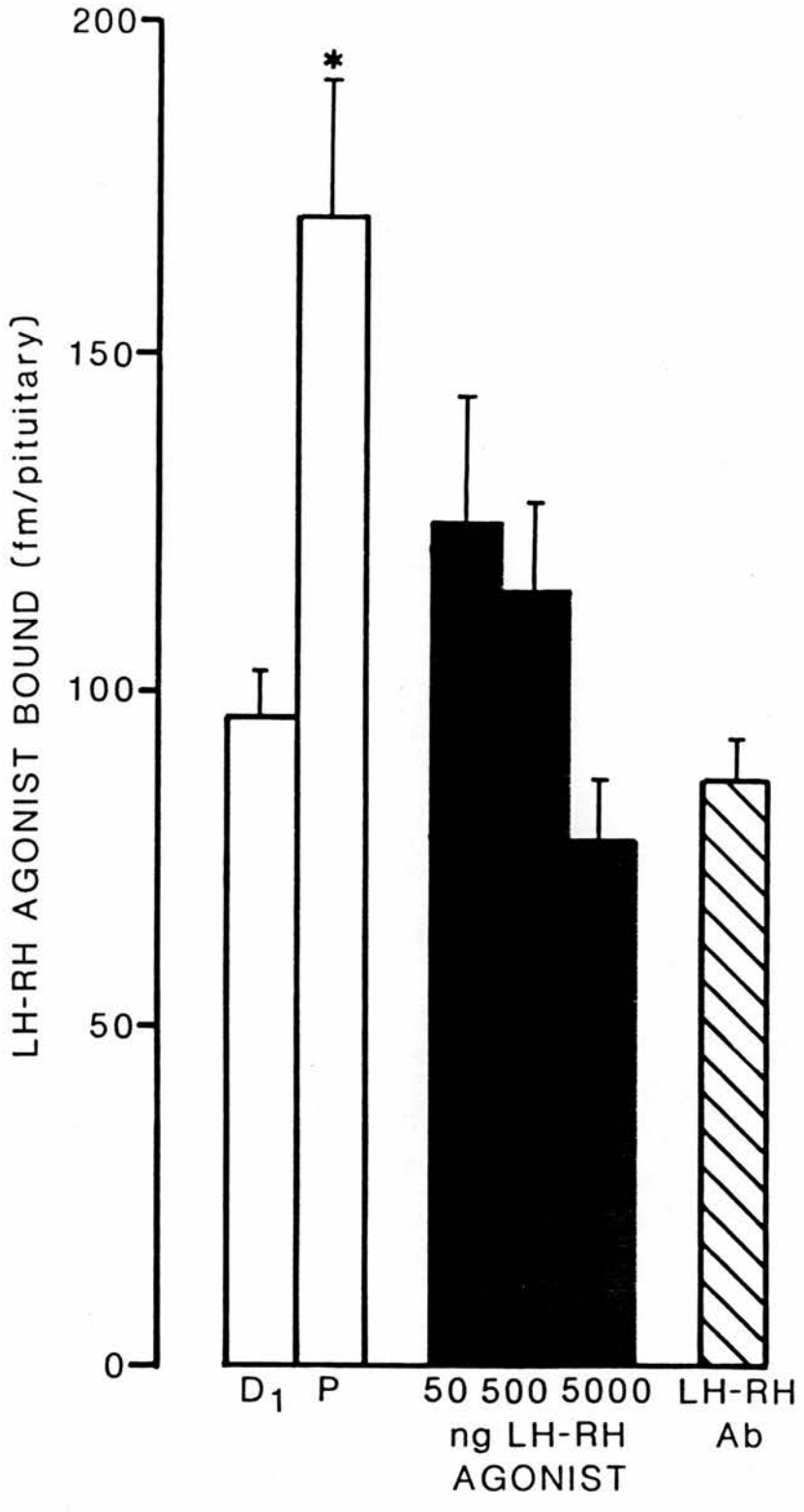

FIGURE 4.7 Effect of daily injections of 50, 500 or 5,000 ng LHRH agonist (■) or 3 times weekly injections of LHRH antibodies (▨) on pituitary LHRH receptors. Control animals received vehicle alone (□) and were killed on the first day of dioestrus (D_1) or pro-oestrus (P).

Values are Mean ± S.E.M. (8 animals per group)

* $P < 0.05$ compared to D_1 control value.

(Group 5). Since no difference was found between HSA and vehicle treated rats, the results were pooled.

24 h after the final injection (or on the day of pro-oestrus or dioestrus for controls in Groups 5 & 6), rats were anaesthetized with CO_2 generated from solid CO_2. Blood was collected by decapitation and after centrifugation the serum was stored at -20°C, prior to hormone assay. The anterior pituitary was rapidly removed and used immediately for the LHRH receptor assay (see Chapter 3.1.3). The data are expressed as mean $\pm$ SEM and values were compared to control dioestrous values (Group 6).

Results: Changes in ovarian histology and receptors are presented in Chapter 5.

LHRH Antiserum Treatment: Hypostimulation of the pituitary following immunoneutralization of endogenous LHRH with a specific LHRH antiserum resulted in significantly decreased serum LH and progesterone concentrations (Table 4.3). Ovarian weight was reduced (Table 4.3). Oestrous cycles, as assessed by vaginal cytology ceased, as evidenced by a constant dioestrous-type vaginal smear. Pituitary LHRH receptors were not statistically different from those of dioestrous control rats (Fig. 4.7).

LHRH agonist treatment: Hyperstimulation of the pituitary using daily treatment with 50, 500 or 5000 ng LHRH agonist, resulted in decreased uterine weight and serum oestradiol (Groups 2 and 3). Serum progesterone was reduced in all groups. Ovarian weight was significantly increased in Groups 1 and 2. Vaginal cytology revealed a constant dioestrous pattern indicative of impaired ovarian

function.

Serum LH was unaltered although serum FSH levels were increased in all treated groups.

Pituitary LHRH receptor concentrations were not significantly different from dioestrous control values (Fig. 4.7).

Discussion: Hyperstimulation of the pituitary with daily injections of LHRH agonist led to a cessation of normal oestrous cycles, as shown by constant dioestrous smears and decreased serum progesterone and oestradiol levels indicative of impaired ovarian function. This agrees with the findings of Johnson et al., (1976a); Cusan et al., (1979) and Maynard & Nicholson, (1979) and provides another example of the paradoxical antifertility effects seen after prolonged administration of LHRH agonist, as discussed in Chapter 1. Receptor changes following LHRH administration as studied in the male (see 4.3.3) have shown that chronic administration of LHRH does not invariably result in decreased receptor numbers. This indicates that the observed antifertility effects (Chapter 1) cannot be explained entirely by a down-regulation of pituitary LHRH receptors under these conditions. Similarly, in the female, decreased pituitary-ovarian function occurred in the absence of receptor changes (Fig. 4.7). Marchetti et al., (1982) found that injection of 200 ng LHRH agonist/day for 2 weeks also failed to alter receptor numbers significantly from those found in intact female controls. The data presented confirms and extends this finding by showing that chronic treatment of intact female rats with a range of doses of LHRH agonist (50, 500 or 5000 ng/day) resulted in pituitary receptor

levels within the range for the cyclic animal. It is unlikely therefore that pituitary LHRH receptor down-reguation is a primary mechanism involved in the antifertility effects seen in either male or female animals after repeated daily injections of LHRH agonist.

Although basal LH levels were unchanged by LHRH agonist administration (see Table 4.3), each injection would be followed by a huge rise in serum LH (Kledzik et al., 1978; Cusan et al., 1979). Thus the possibility of gonadal desensitization following excess gonadotrophin stimulation (Conti et al., 1976) cannot be excluded. Neither can direct ovarian actions of LHRH agonist.

Immunoneutralization of LHRH also led to cessation of oestrous cycles and impaired ovarian function similar to that seen after active immunization against LHRH (Fraser & Baker, 1978). Despite this, pituitary LHRH receptors were within the range found in control cycling animals at dioestrus (Fig. 4.7). Since passive immunization of intact males with the same antiserum resulted in a 25-35% reduction in LHRH receptors (see 4.3.3.3) it was surprising that no such effect was seen in the intact female. However, it must be remembered that receptor numbers vary during the oestrous cycle and these cyclic changes presumably do not occur under the treatment regime. Thus it could be suggested that after 3 weeks of antibody treatment the presence of ovarian steroids, albeit at altered levels, is sufficient to maintain receptor levels at the basal cyclic level.

Alternatively, it is possible that prolonged administration of LHRH antiserum resulted in the development of antibodies to the injected antiserum and thus complete immunoneutralization of LHRH

cannot be assumed.

It is also possible, but unlikely, that a fundamental difference exists between males and females in the importance of pituitary LHRH receptor autoregulation in the intact animal.

Conclusions:

1) Prolonged daily hyperstimulation of the pituitary with LHRH agonist disrupted oestrous cycles but did not result in altered pituitary LHRH receptor concentrations, indicating an absence of pituitary receptor down-regulation.

2) Hypostimulation also failed to alter pituitary LHRH receptors from the range seen in the cycling rat.

3) Therefore, either the role of autoregulation is secondary to the influence of circulating hormones in the intact animal, or the regime used was insufficient to neutralize all endogenous LHRH.

4.4.3 Active immunization against LH and LHRH

Introduction: In order to assess the relative contribution of LH, steroids and LHRH in the regulation of pituitary LHRH receptors in the female, the effects of active immunization against LH and LHRH were compared.

Method: 31 regularly cycling adult female Sprague Dawley rats were immunized as described in Chapter 2.6. 14 control rats were immunized against BSA, 6 rats against LH and 11 against LHRH. Three months after the initial injections, rats were boosted using Freund's incomplete adjuvant, then killed 3 weeks later, blood and pituitaries being collected as described previously. Ovaries were also removed for histology and assessment of LHRH receptor concentration (Chapter 5).

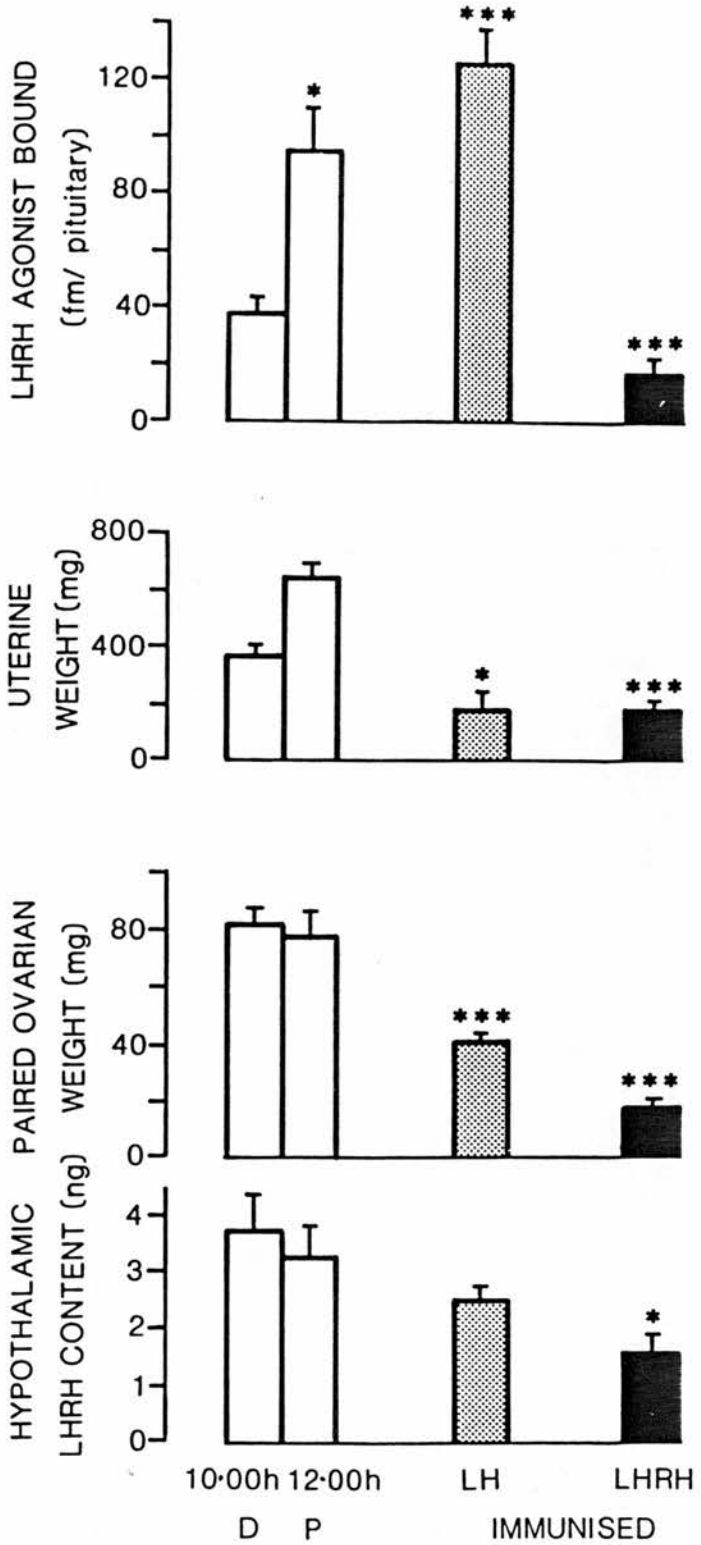

FIGURE 4.8 Effect of active immunization of adult female rats against LH (▨) or LHRH (■) on pituitary LHRH receptors, uterine and ovarian weight and hypothalamic LHRH content. Control animals were immunized against BSA and killed either at 10.00 h dioestrus (D) or 12.00 h pro-oestrus (P).

Data represents Mean + S.E.M. (7 in each of control groups, 11 LHRH immunized and 6 $\overline{LH}$ immunized).

* $P < 0.05$; *** $P < 0.001$ compared to D control value.

Results: Out of 14 control rats, 2 were spontaneously acyclic and 2 showed cycles of abnormal length so the tissues were not included in the receptor assays. These results are unlikely to be due to control immunization procedure since a group of 12 untreated rats of similar age (200 days old) showed similar characteristics. The remaining control rats exhibited regular 4 day cycles and were killed either at 10.00 h dioestrus or at noon pro-oestrus.

Although LH-antibody titres were not measured, they were clearly sufficient to abolish ovarian function in all animals, as evidenced both by cessation of oestrous cycles, and dramatically decreased ovarian uterine weight as previously described by Lawrence & Ichikawa (1968). Pituitary LHRH receptors were significantly elevated in the LH-immunized rats, compared with dioestrous control levels, to levels comparable to those found at pro-oestrus in the cycling animal (Fig. 4.8).

LHRH antibody titres ranged from 1:900-1:13,000 and all rats showed characteristic changes associated with immunoneutralization of LHRH, namely decreased uterine weight, cessation of cyclicity (as assessed by vaginal smears) and decreased ovarian weight (Fraser & Baker, 1978). These changes were similar to those seen after LH immunization. However, in marked contrast to LH, immunization of LHRH resulted in dramatic reduction in the number of pituitary LHRH receptors ($P \leq 0.001$) and hypothalamic content of LHRH (Fig. 4.8).

Discussion: The ability of chronic immunoneutralization of LHRH to reduce pituitary LHRH receptors in the intact female indicates that

as in the male, autoregulation is involved in the maintenance of pituitary LHRH receptors.

The receptor changes following active immunization against LH were similar to those seen after ovariectomy (see 4.4.4) and indicate that the removal of gonadal steroids raises receptor levels to those found in pro-oestrous rats.

Conclusions:

1) Gonadal steroids exert a negative influence on pituitary LHRH receptors.

2) LHRH receptors are positively autoregulated in the intact animal.

4.4.4 Passive Immunization of LHRH in ovariectomized rats

Introduction: Extensive studies in the male had indicated that the post-castration LHRH receptor increase was due to LHRH autoregulation (discussed in 4.3.3.4). In order to assess the efficacy of the LHRH antiserum in females, and to determine the role of autoregulation in post-ovariectomy receptor changes, the same antiserum which had successfully immunoneutralized endogenous LHRH in the male (4.3.3.3), was administered to female rats 7 days after ovariectomy.

Method: 12 adult female rats, at random stages of the oestrous cycle were ovariectomized under ether anaesthesia. After seven days the animals were injected intravenously under ether anaesthesia, with either 0.5 ml LHRH antiserum (94) or HSA antiserum. 21 h later blood and anterior pituitary glands were collected as described previously.

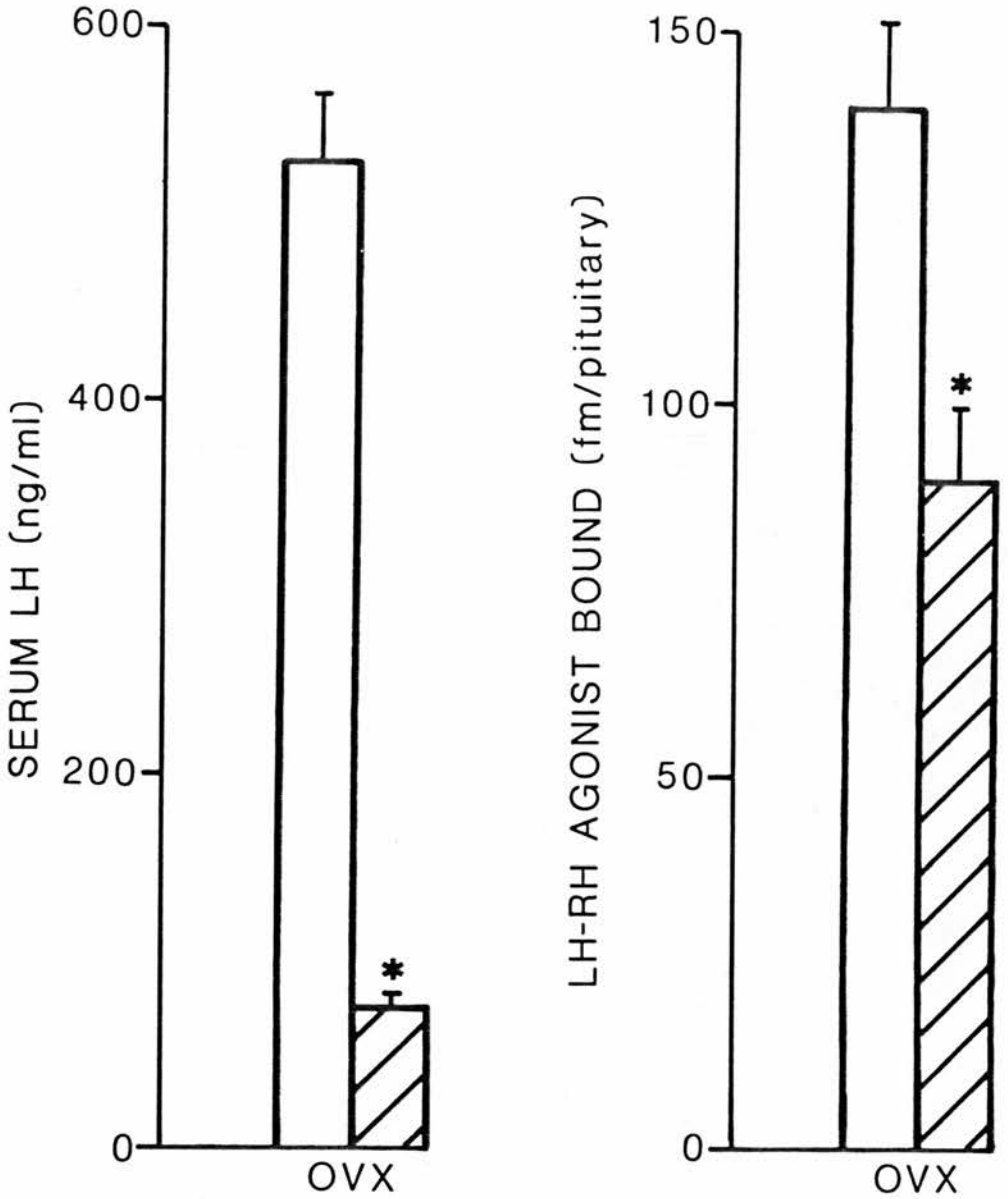

FIGURE 4.9 Effect of administration of LHRH antiserum to adult female rats ovariectomized 7 days previously, on pituitary LHRH receptors and serum LH measured 21 hours later.

Data are Mean ± S.E.M. (6 animals per group).

* $P < 0.05$.

Results: Ovariectomy resulted in elevated serum LH levels as measured 8 days later and these levels were reduced ($P < 0.05$) by treatment with LHRH antiserum 21 h previously (Fig. 4.9). Pituitary LHRH receptors were raised to pro-oestrous levels in the control ovariectomized rats while after LHRH antibody treatment, receptor numbers fell ($P < 0.05$) to the range found for oestrous rats (Fig. 4.9).

Discussion: The ability of specific immunoneutralization of endogenous LHRH to reduce the post-ovariectomy rise in LHRH receptors emphasises the importance of endogenous LHRH in maintaining LHRH receptors after castration. This has also been found to be the case in males (e.g., Clayton, 1982).

4.4.5 The effects of passive immunoneutralization of LHRH or LH on Pituitary LHRH receptors during the rat oestrous cycle.

Introduction: Pituitary LHRH receptor numbers vary during the oestrous cycle of the rat and hamster with a slow maintained increase during dioestrus day one, reaching maximal levels between dioestrus day 2 and the morning of pro-oestrus, after which numbers fall to a nadir at oestrus (e.g. Adams & Spies, 1981a; Clayton et al., 1980; Savoy-Moore et al., 1980). The increase in receptors has been suggested to be influenced either by ovarian steroids and/or gonadotrophins, or an autoregulatory action of LHRH (Savoy-Moore et al., 1980; Clayton, 1982). The reasons for the fall in LHRH receptors are largely unknown, but could be due to LHRH down-regulation similar to that described for LH (Conti et al., 1976), since portal blood levels of

LHRH are raised on the afternoon of pro-oestrus (Sarkar et al., 1976; Levine & Ramirez, 1982). Alternatively or additionally the changing steroid environment could be involved.

One way to investigate the factors stimulating these changes within the 'LHRH-LHRH receptor-LH-ovarian steroid axis' is to specifically neutralize either LHRH or LH by antisera. Injection of antibodies to LHRH on the first day of dioestrus prevents follicular development and oestrogen secretion, while administration at noon on pro-oestrus prevents the LH surge and ovulation (Fraser & Gunn, 1973; Koch et al., 1973; de la Cruz et al., 1976). Injection of LH antibodies at these times has similar effects on ovarian function, but leaves LHRH free to act on its pituitary receptors (Schwartz & Ely, 1970; Ely & Schwartz, 1971).

Materials and Methods: Adult female Sprague Dawley rats (60-80 days old) housed under conventional conditions with lights on between 05.00 h and 19.00 h and with regular 4 day oestrous cycles, were used in the study.

Antisera: The LHRH antiserum (No. 94) had been used previously in males (4.3.3.3) and females (4.4.2). Preliminary results showed that the minimum effective dose of the LHRH antiserum required to block ovulation in 5 out of 5 rats when administered intravenously at 12.00 h pro-oestrus, was 100 µl. A dose of 0.5 ml was used in the main study.

The LH antiserum was raised against ovine LH (NIAMDD-oLH-23) in a New Zealand White Rabbit (Sharpe & Fraser, 1983). Antiserum obtained from one animal (R28) after the first booster immunization,

was used in the present study. Titration curves prepared by incubating dilutions of the antiserum under standard radioimmunoassay conditions showed that the titres of the antiserum, expressed as the final dilution binding 33% of ^{125}I-oLH or ^{125}I-rLH, were 1:1,350,000 and 1:195,000 respectively. The antiserum also bound ^{125}I-rFSH, but at a much lower titre of 1:9,600. Initial studies showed 250 µl of this antiserum blocked ovulation in 5 out of 5 rats when administered intravenously at 12.00 h pro-oestrous.

Administration of antisera at 11.00 h Dioestrus: To examine the effects of neutralization of LHRH or LH on the rise in pituitary LHRH receptor numbers between the first day of dioestrus and noon pro-oestrus, 3 groups of 5 rats were injected intravenously via the tail vein (under light ether anaesthesia) at 11.00 h dioestrus one, with either 0.5 ml LHRH antiserum, 1 ml LH antiserum or 1 ml antiserum to human serum albumin (HSA). 5 untreated control rats were killed with CO_2 generated from dry ice at 11.00 h dioestrus one. The antibody treated rats were killed at 12.00 h pro-oestrus, with blood and serum collected as described previously. The uterus from each animal was weighed and checked for distension.

Administration of antisera at 12.00 h Pro-oestrus: To examine the effects of immunoneutralization of LH or LHRH on the decrease in pituitary LHRH receptors between 12.00 h pro-oestrus and 09.00 h oestrus, 2 groups of 10 rats were injected at 12.00 h pro-oestrus with either 0.5 ml LHRH antiserum, or 0.5 ml HSA antiserum. 5 untreated control rats were killed at 12.00 h pro-oestrus. 5 rats

from each group were killed at 17.00 h pro-oestrus. The remaining animals were bled from the tail vein at 17.00 h pro-oestrus (under light ether anaesthesia) and killed at 09.00 h oestrus. 5 rats were injected with 1 ml LH antiserum at 12.00 h pro-oestrus and killed at 09.00 h oestrus. Blood, pituitaries and right ovaries were collected as described previously. Oviducts were removed from animals killed at oestrus and examined for ova.

Results: (Data on ovarian receptors is presented in Chapter 5).

Immunoneutralization of LH or LHRH at 11.00 h dioestrus one

The effectiveness of the antisera to LHRH or LH in blocking follicular steroidogenesis was demonstrated by the fact that the increase in uterine weight ($P < 0.001$) between dioestrus and pro-oestrus observed in control animals, was completely prevented ($P < 0.001$) in the rats receiving LHRH or LH antibodies (Fig. 4.10). While all the control animals showed evidence of uterine distension, this was absent in all the treated rats.

Control animals showed the expected increase in pituitary LHRH receptors between 11.00 h dioestrus one and 12.00 h pro-oestrus ($P < 0.001$; Fig. 4.10). Passive immunoneutralization of LHRH prevented this receptor increase ($P < 0.001$; Fig. 4.10) whereas administration of LH antiserum had no effect (Fig. 4.10).

Immunoneutralization of LH or LHRH at 12.00 h pro-oestrus

The effectiveness of passive immunization of LHRH or LH at 12.00 h pro-oestrus was indicated by the abolition of ovulation in animals killed at 09.00 h oestrus. Control animals shed an average of 14 ova (range 12-16) whilst oviducts of LHRH and LH antiserum

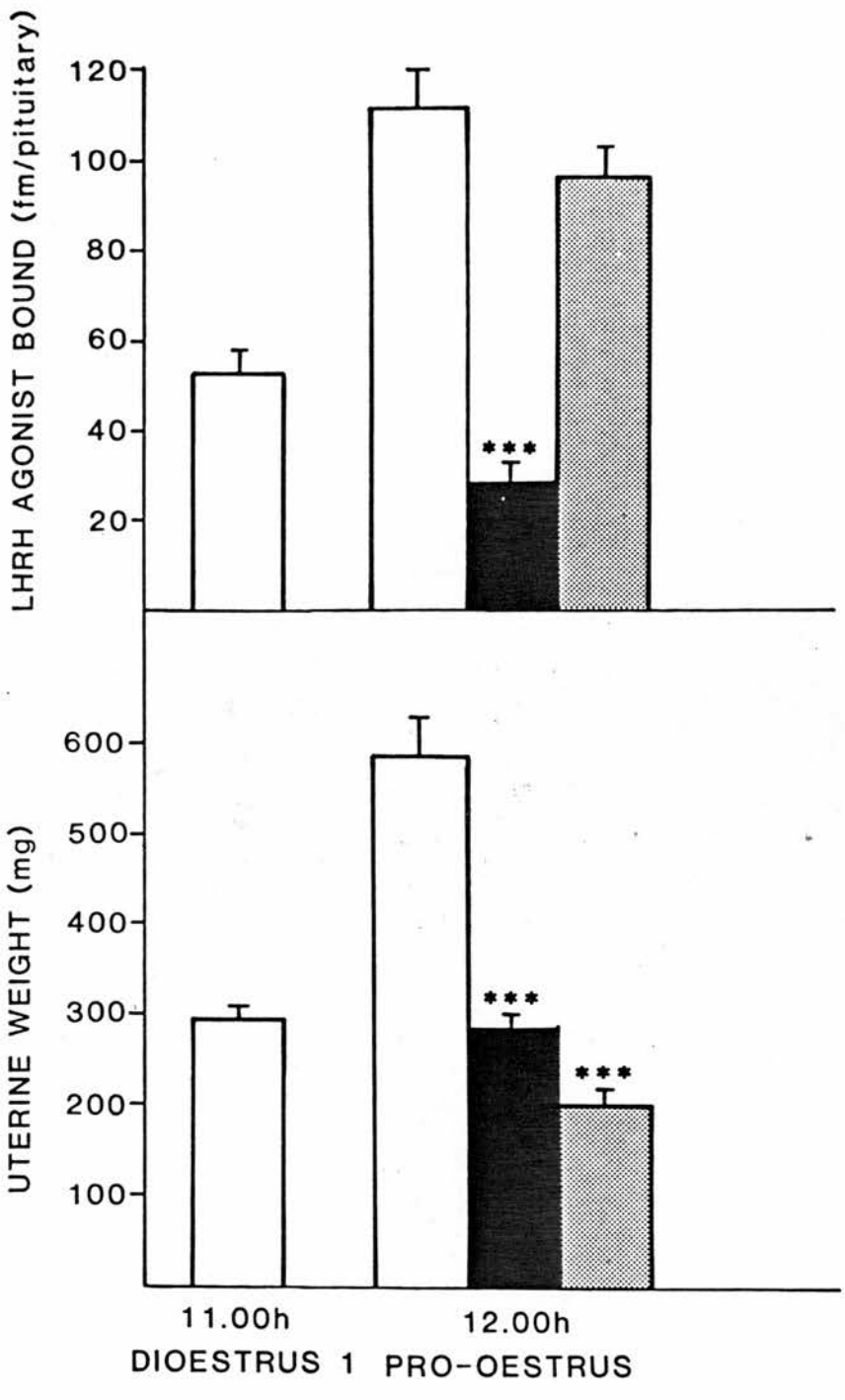

FIGURE 4.10 Effect of administration of antiserum to LH (▨), LHRH (■) or control serum (□) at 11.00 h dioestrus on pituitary LHRH receptors and uterine weight measured at 12.00 h pro-oestrus.

Data are Mean ± S.E.M. (5 animals per group).

*** $p < 0.001$ for antiserum treated compared to pro-oestrous control value.

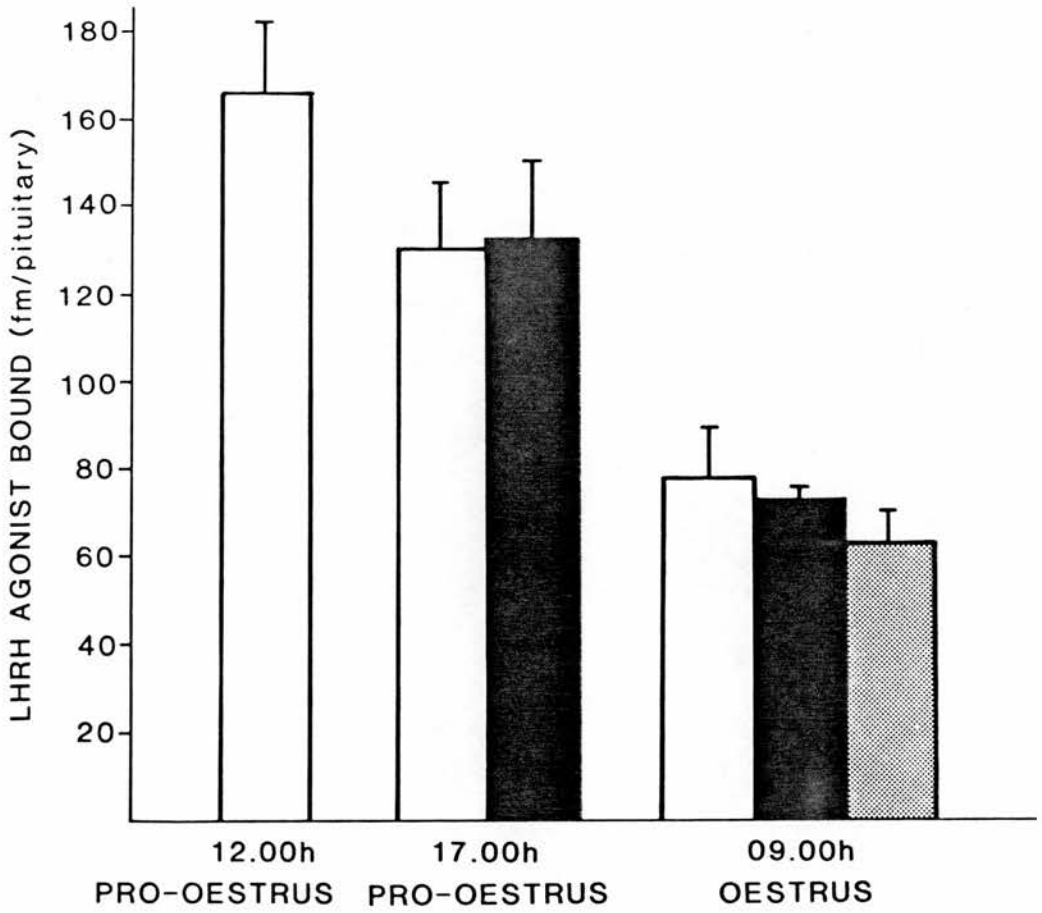

FIGURE 4.11 Effect of administration of antiserum to LH (▨), LHRH (■) or HSA (□) at 12.00 h pro-oestrus, on pituitary LHRH receptors measured at 17.00 h pro-oestrus and 09.00 h oestrus.

Data are Mean ± S.E.M. (5 animals per group).

treated animals had no ova present. Passive immunization of LHRH also abolished the LH surge, reducing serum LH levels from 250±60 ng/ml (control) to 36±3 ng/ml (antiserum treated) at 17.00 h pro-oestrus ($P < 0.05$).

Pituitary receptors declined from 12.00 h pro-oestrus to 09.00 h oestrus in control animals ($P < 0.001$; Fig. 4.11). Administration of LHRH or LH antiserum at 12.00 h pro-oestrus did not alter the receptor numbers (Fig. 4.11); neither were receptor numbers altered at 17.00 h pro-oestrus in control or LHRH antiserum treated animals.

Discussion: The neutralization of LHRH or LH by antibodies from the first day of dioestrus had similar effects, both treatments blocking follicular oestrogen secretion as indicated by the absence of increased uterine weight on the morning of pro-oestrus. However, these treatments had markedly different effects on the rise in the number of pituitary LHRH receptors which occurs during this period (Park et al., 1976; Clayton et al., 1980; Savoy-Moore et al., 1980; Marian et al., 1981; Reeves et al., 1982). While the LH antiserum failed to effect this change, treatment with LHRH antiserum completely abolished the rise. Since the essential difference between the inhibitory treatments on the 'LHRH-LHRH receptor-LH- ovarian steroid axis' is the removal of the LHRH by the LHRH antibodies, these results suggest that the primary driving force for the rise in pituitary LHRH receptors at pro-oestrus is LHRH itself.

Pituitary receptors showed a marked fall from high levels at 12.00 h pro-oestrus to low levels at 09.00 h oestrus, a finding in

agreement with that of others (Park et al., 1976; Savoy-Moore et al., 1980; Clayton et al., 1980). Since LHRH levels in hypophyseal portal blood are highest during the afternoon of pro-oestrus (Sarkar et al., 1976; Levine & Ramirez, 1982) it is possible that LHRH itself acts as the stimulus for its own receptor decline. Immunoneutralization of LHRH at 12.00 h pro-oestrus resulted in abolition of the LH surge and ovulation. Despite this, the number of pituitary LHRH receptors was unaltered at 17.00 h pro-oestrus and the fall to 09.00 h oestrous levels was not prevented. Since LHRH antibodies presumably prevent LHRH reaching its pituitary receptor, the inability of immunoneutralization of LHRH to influence the receptor decline indicates that the change in the number of pituitary receptors between pro-oestrus and oestrus is independent of LHRH. Immunoneutralization of LH similarly resulted in abolition of ovulation and also failed to influence the receptor decline. The low receptor numbers at 09.00 h oestrus are therefore likely to be due to changes occurring between 12.00 h pro-oestrus and 09.00 h oestrus, which are independent of changes in the secretion of LHRH, LH or ovarian steroids during this period. It has been suggested that the low receptor numbers at oestrus are due to occupation by endogenous ligand and therefore do not represent a true fall (Smith-White & Ojeda, 1983). However, this is unlikely to be the case due to the rapid rate of dissociation of LHRH from its hormone receptor complex under the conditions of the assay (Clayton & Catt, 1981b).

Detailed time course studies of receptor changes between pro-oestrus and oestrus have indicated that 2 phases may be involved;

an initial acute fall which is transitory, followed by a more gradual decrease to nadir levels (Savoy-Moore et al., 1980; Adams & Spies, 1981a). The acute fall was abolished by pentobarbitone given at 12.00 h pro-oestrus (Savoy-Moore et al., 1981; Adams & Spies, 1981b) and in the hamster could be restored by LHRH administration (Adams & Spies, 1981b). A similar acute fall was observed in the ovariectomized oestrogen-treated rat which was also pentobarbitone sensitive (Barkan et al., 1983b). Although this acute transitory fall appears to be LHRH dependent, several problems exist concerning interpretation of its relevance. Firstly, it appears to occur prior to the LH surge (Savoy-Moore et al., 1981; Barkan et al., 1983b) and secondly, it may be modulated by endogenous opioids (Barkan et al., 1983a) by a mechanism independent of endogenous LHRH, since reduced portal blood LHRH was not always associated with the impaired LH surge following treatment with naloxone or morphine (Ching, 1983). In addition it may represent an apparent loss of receptors only, due to receptor occupancy (Smith-White & Ojeda, 1983). In this study we investigated the overall decrease in receptors which occurred between pro-oestrus and oestrus and which seemed to be unaffected by pentobarbitone (Savoy-Moore et al., 1981) and was unlikely to be due to receptor occupancy (Smith-White & Ojeda, 1983).

Since pentobarbitone also influences the release of other hormones it was important to establish whether the overall fall in receptors would be affected by specific neutralization of LHRH. Our data extends those obtained using pentobarbitone and indicates that the fall in receptors is likely to be independent of LH, LHRH and

ovarian steroids and is therefore probably under the control of other factors of hypothalamic or ovarian origin. Alternatively, the return of receptors to basal levels may be a pre-programmed response to hormonal changes which occurred earlier in the cycle.

In addition, it could be postulated that since specific removal of LHRH has been shown to result in decreased LHRH receptors (e.g., see 4.3.3.3 and 4.4.3), the decrease in LHRH receptors at pro-oestrus is due to a removal of endogenous LHRH support, rather than down-regulation following high LHRH stimulation. Thus portal blood LHRH concentrations are known to be maximal coincident with the LH surge, falling to nadir levels on the evening of pro-oestrus (Sarkar et al., 1976; Levine & Ramirez, 1982). In conclusion, it appears that:-

(1) LHRH autoregulation alone is responsible for the increase in pituitary LHRH receptors between dioestrus one and noon pro-oestrus, but that

(2) the fall in receptors from pro-oestrus to oestrus is independent of LHRH, LH and ovarian steroids. Whether the fall is regulated by a pre-programmed mechanism, or by other factors such as endogenous opioids, remains to be determined.

4.4.6 LHRH Receptor Regulation in the Female - Summary

The experimental results obtained from the studies described in the female are summarized in Table 4.4.

The results show:-

(1) Chronic hyperstimulation of the pituitary with LHRH agonist failed to down-regulate pituitary LHRH receptors.

TABLE 4.4

Summary of data obtained from studies on pituitary LHRH receptor regulation in the female.

Arrows indicate the direction of known or assumed changes relative to dioestrous control values.

Treatment	LHRH Stimulation of Pituitary	LHRH Receptors	Serum LH	Steroids E_2 P
LHRH agonist - 3 weeks	↓↓	No change	No change	↓
LHRH antiserum - 3 weeks	↓ ?	No change	↓	No change ↓
Active Immunization against LH	?	↑	↓↓↓	↓↓
Active Immunization against LHRH	↓↓	↓↓	↓↓	↓↓
Passive Immunization against LH	?	No change	↓↓	↓
Passive Immunization against LHRH	↓↓	↓	↓	↓
Ovariectomy	? ↑	↑	↑	Non detectable
Ovariectomy + LHRH antiserum	↓	↓	↓	Non detectable

(2) Active immunization against LH resulted in increased receptor concentrations.

(3) Active immunization against LHRH resulted in decreased receptor concentrations.

(4) Passive immunization against LHRH decreased the high post-ovariectomy receptor numbers.

(5) Passive immunization against LH had no effect on pituitary receptor numbers during the oestrous cycle.

(6) Passive immunization of LHRH in intact female rats at dioestrus prevented the increase in receptors at pro-oestrus, but administration at noon pro-oestrus did not prevent the fall in receptor numbers at oestrus.

Conclusions:

(1) Pituitary LHRH receptor down-regulation is unlikely to be the sole explanation for the antifertility effects of LHRH agonist.

(2) Pituitary LHRH receptors are primarily under positive auto-regulatory control, numbers reflecting known or assumed changes in endogenous LHRH release.

(3) The fall in receptor numbers between pro-oestrus and oestrus is unlikely to be due to LHRH receptor down-regulation and may be caused by removal of positive autoregulatory influence of LHRH i.e. decreased LHRH release.

(4) Positive autoregulatory control of LHRH receptors is physiologically important in inducing the increase in receptor concentration at pro-oestrus.

CHAPTER 5

REGULATION OF OVARIAN LHRH RECEPTORS

CHAPTER 5

REGULATION OF OVARIAN LHRH RECEPTORS

5.1 INTRODUCTION AND AIMS

5.2 OVARIAN LHRH RECEPTORS - REVIEW

5.3 OVARIAN LHRH RECEPTORS AND MORPHOLOGY IN RATS EXPOSED TO CONSTANT LIGHT

5.3.1 Method
5.3.2 Results
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5.4 OVARIAN LHRH RECEPTORS AND MORPHOLOGY FOLLOWING CHRONIC TREATMENT WITH LHRH ANTISERUM OR LHRH AGONIST

5.4.1 Method
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5.5 OVARIAN LHRH RECEPTORS AND MORPHOLOGY IN RATS ACTIVELY IMMUNIZED AGAINST LH OR LHRH

5.5.1 Method
5.5.2 Results
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5.6 OVARIAN LHRH RECEPTORS IN CYCLIC RATS PASSIVELY IMMUNIZED AGAINST LH OR LHRH

5.6.1 Method
5.6.2 Results
5.6.3 Discussion

5.7 CONCLUSIONS

5.8 GENERAL DISCUSSION

5.1 INTRODUCTION AND AIMS

From the data discussed in the previous chapter it is evident that pituitary LHRH receptors are regulated by both heterologous factors, such as prolactin and steroids, and by LHRH itself. Since ovarian LHRH receptors have been shown to have identical characteristics of affinity and specificity to those of the pituitary (Chapter 3) it seemed reasonable to expect that similar regulatory processes could be applied.

The experiments detailed in this chapter therefore set out to determine whether ovarian LHRH receptor concentrations, measured in whole ovarian homogenates (as described in Chapter 3) varied under conditions known to produce marked alterations in ovarian structure and function, namely:-

(a) Exposure to constant light

(b) Active immunization against LH or LHRH

(c) Chronic hyperstimulation with LHRH agonist or hypostimulation following prolonged passive immunoneutralization of LHRH

(d) Short term passive immunization against LH or LHRH.

Experimental details, together with serum analyses and pituitary LHRH receptors, are presented in Chapter 4; only the ovarian receptors are discussed in this chapter. For each experiment the ovaries were rapidly removed and either stored at -40°C prior to assay of LHRH receptors or taken for histology. No differences were found between right and left ovaries which were taken at random for receptor assay or histology.

5.2 OVARIAN LHRH RECEPTORS - REVIEW

Gonadal LHRH receptor concentrations show marked changes during the period of sexual maturation in both male and female rats, with receptor concentrations being highest in immature rats (Harwood et al., 1980b). Receptor levels were low at day 5 of age and rose to maximal at day 20-25 (Dalkin et al., 1981; Smith-White & Ojeda, 1981) before declining to adult levels by day 40-80 of age (Dalkin et al., 1981). This prepubertal decline in LHRH receptor concentrations occurred during the days preceeding the first pre-ovulatory LH surge (Smith-White & Ojeda, 1981). These receptor changes during sexual maturation bear considerable resemblance to those reported for the pituitary (Chapter 4.2.1). In both cases, expression of receptor numbers per gland shows gradual increase with increasing age and tissue weight (Dalkin et al., 1981).

In contrast to the pituitary, total ovarian LHRH receptor concentrations do not alter during adulthood in female rats, no evidence being available for cyclic changes during the oestrous cycle (Dalkin et al., 1981; Pieper et al., 1981; Reeves et al., 1982). However ovarian LHRH receptor concentrations were found to decrease in the third week of pregnancy and in the first week of lactation (Reeves et al., 1982).

As with pituitary LHRH binding, there was no evidence to suggest an alteration in ovarian LHRH receptor affinity (Dalkin et al., 1981; Reeves et al., 1982), thus validating the use of saturation analysis for receptor measurement (Chapter 3) in the following studies.

5.3 OVARIAN LHRH RECEPTORS AND MORPHOLOGY IN RATS EXPOSED TO CONSTANT LIGHT

5.3.1 Method

Ovarian LHRH receptors were assessed in individual ovaries (as described in Chapter 3) taken from adult female rats kept under conditions of constant light (350 - 400 lux) for 14 weeks and compared with those of 10 normal adult rats of the same age killed at dioestrus or pro-oestrus (details given in 4.2.5).

5.3.2 Results

Vaginal smears revealed a predominance of cornified cells typical of a persistent oestrous state induced by exposure to constant light. Ovarian histology indicated a marked absence of active luteal tissue and the ovaries showed the typical polyfollicular appearance indicative of impaired ovarian function (Plate 5.1 and see also Lawton & Schwartz, 1967; Reiter & Klein, 1971). Despite these changes in ovarian morphology, serum LH and oestradiol concentrations have been reported as being within the normal cyclic range in rats exposed to constant light (Naftolin et al., 1972; Brown-Grant et al., 1973).

Ovarian LHRH receptor concentrations were significantly higher than dioestrous control values ($P < 0.001$; Fig. 5.1). In addition, no difference was observed in receptor concentrations (or numbers per gland) between dioestrous and pro-oestrous control ovaries.

Ovarian weight was decreased by treatment (control ovaries combined weight 80±7 mg: constant light ovaries 41±4 mg; $P < 0.001$; mean ± S.E.M.). Expression of the receptor data as fm LHRH agonist bound/ovary abolished the increase seen after exposure to constant light (Fig. 5.1).

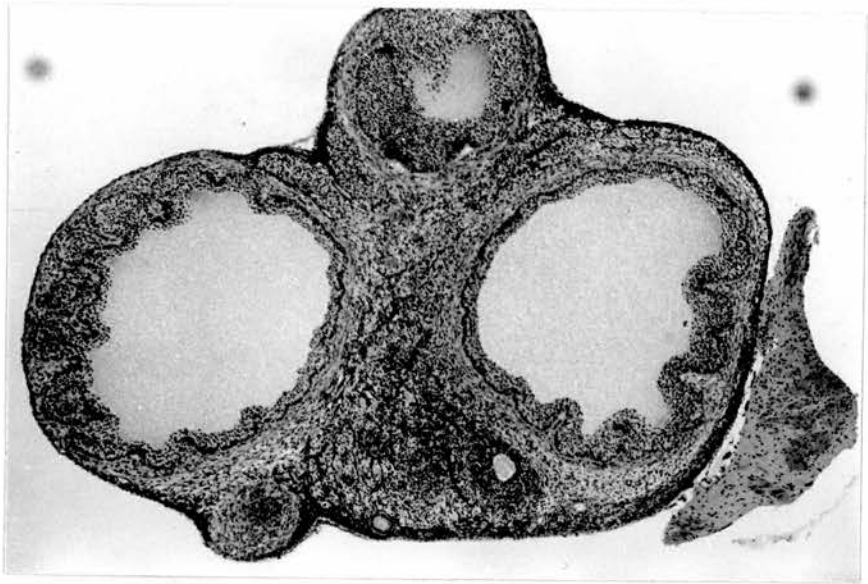

PLATE 5.1 Histological section through rat ovary from a rat kept under conditions of constant light. X 50.

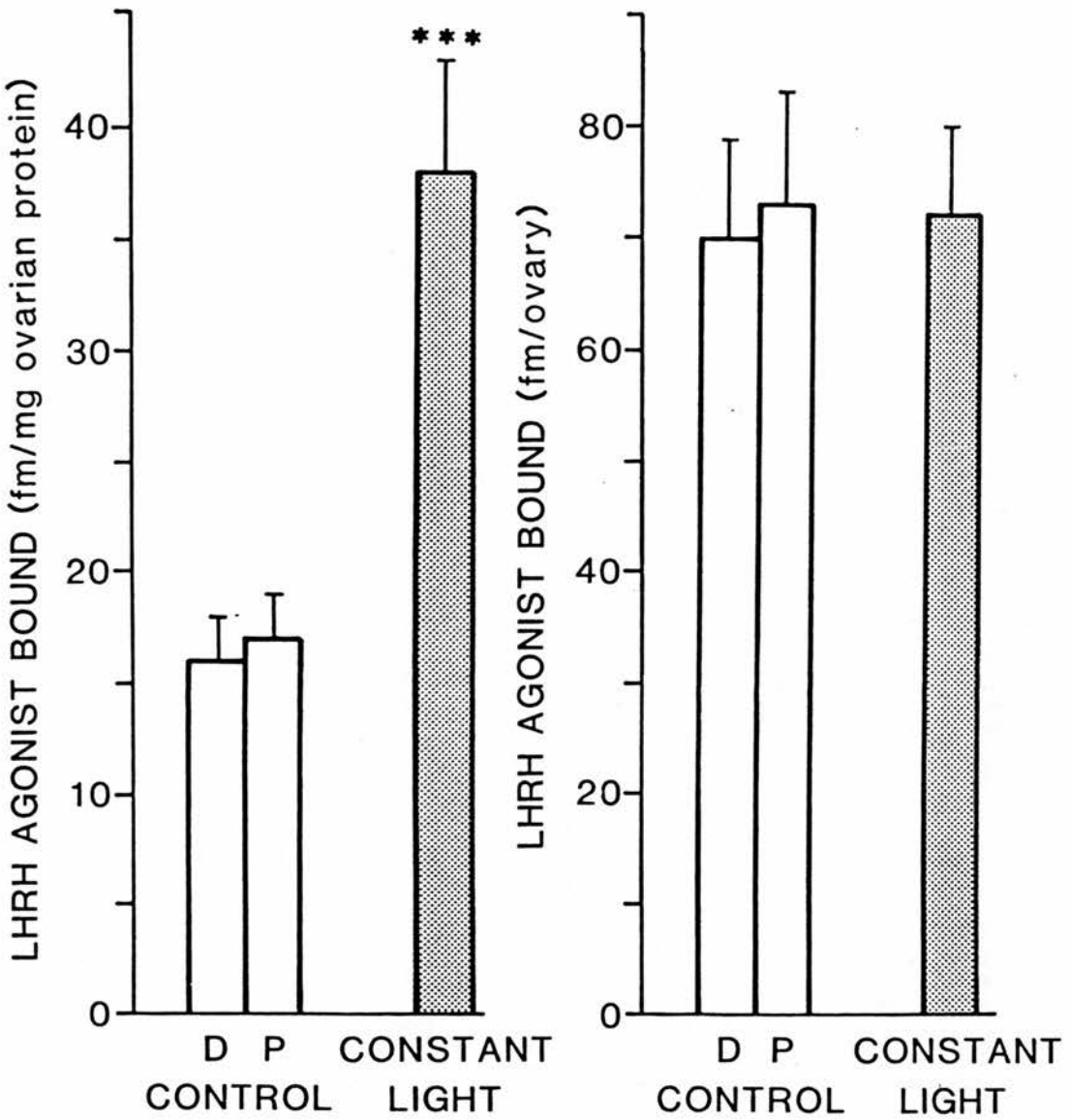

FIGURE 5.1 Ovarian LHRH receptors measured in control rats (□) at 10.00 h dioestrus (D) or 12.00 h pro-oestrus (P) and in rats exposed to constant light (▦).

Data are expressed as fm bound per mg ovarian protein (left panel) and as fm bound per ovary (right panel) and represent Mean ± S.E.M. (10 constant light rats and 5 rats in each control group).

*** $P < 0.001$ for constant light compared to control D value.

5.3.3 Discussion

In contrast to the pituitary, ovarian LHRH receptors from control adult cyclic rats were unchanged at dioestrus and pro-oestrus, indicating an absence of cyclic variation of receptor concentrations in the whole rat ovary (see also Dalkin et al., 1981; Reeves et al., 1982).

Although pituitary LHRH receptors were within the normal cyclic range after exposure to constant light (see 4.2.5), ovarian receptor concentrations were raised. This may be a reflection of the reported higher concentration of LHRH receptors in follicular compared with luteal tissue (Pieper et al., 1981).

It is evident from the data presented that ovarian and pituitary LHRH receptors are likely to be independently regulated.

5.4 OVARIAN LHRH RECEPTORS AND MORPHOLOGY FOLLOWING CHRONIC TREATMENT WITH LHRH AGONIST OR LHRH ANTISERUM

5.4.1 Method

Adult female rats were treated daily for 3 weeks with 50, 500 or 5,000 ng LHRH agonist (Group 1-3) or LHRH antiserum No. 94 (Group 4) as described previously (4.4.2). Ovaries were removed immediately after cessation of treatment and either taken for LHRH binding assay or placed in Bouin's solution for fixation and histology. This experiment was performed in collaboration with Dr. R. Gosden.

5.4.2 Results

The effects of hypo or hyperstimulation with LHRH on serum gonadotrophins and steroids, on ovarian and uterine weight and on pituitary LHRH receptors are discussed in 4.4.2.

LHRH agonist treatment: Long-term treatment with LHRH agonist

resulted in cessation of oestrous cycles (as evidenced by constant dioestrous type vaginal smears), increased ovarian weight, decreased uterine weight and serum oestradiol (Table 4.3). In addition dramatic alterations in ovarian morphology were evident, ovaries having a predominance of luteal tissue (Plates 5.2 & 5.3). Assessment of the contribution of follicles, corpora lutea and stroma to the overall mass of the ovaries showed the greatest degree of contrast between the 5,000 ng/day group (Group 3) and the controls at dioestrus and pro-oestrus (Groups 6 and 5). Growing follicles (from unilaminar to Graafian sizes) were notably deficient in Group 3 rats and in one animal were virtually absent. These types were also less abundant in the treated animals in Groups 1 and 2, although to a smaller degree. The numbers of primordial (non-growing) follicles were counted in one approximately equatorial section of each ovary but were unaffected by the treatments (range of means 11-15 in Groups 1, 2 and 3). Small and medium-sized follicles appeared to be growing normally whereas Graafian follicles were frequently seen to be undergoing premature luteinization without releasing the oocyte (Plate 5.3). These oocytes had not resumed meiosis, neither was there any mucification of the adjacent cumulus cells, suggesting that the follicles had not been exposed to a normal ovulatory stimulus despite the luteinization of the granulosa cells. Ovaries of rats in groups 1-3 contained many lutein bodies some of which contained a structure which may have been the remnant of an oocyte that had undergone cytoplasmic cleavage (Plate 5.3). These bodies differed therefore from the corpora lutea of the control ovaries, although they were similar in size and in cellular morphology and eosinophilia.

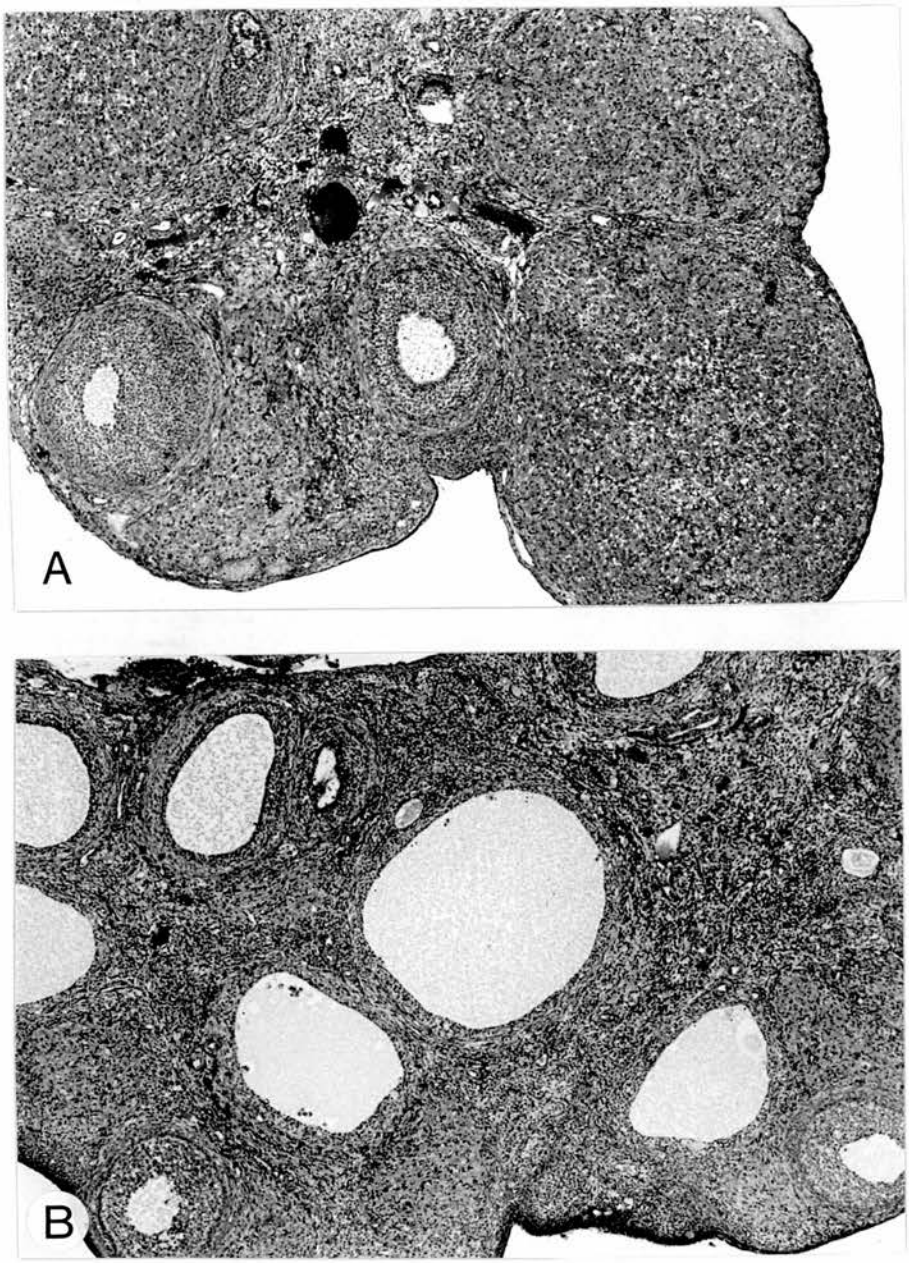

PLATE 5.2 Histological section through rat ovaries from normal dioestrous rats (A) or rats treated with LHRH antiserum (B). X 50.

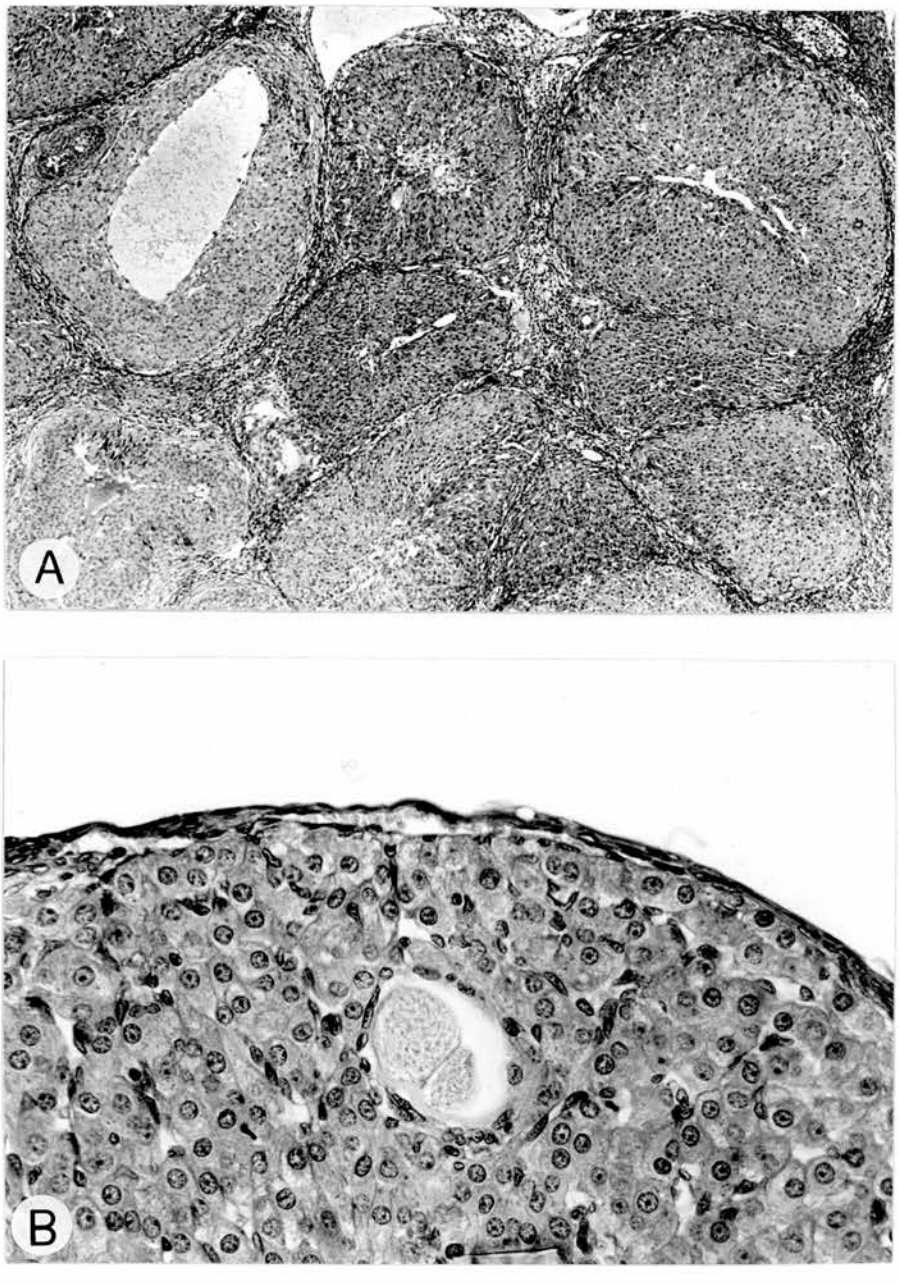

PLATE 5.3 Histological section through rat ovaries from LHRH agonist treated rat showing predominance of luteal tissue (A) X 50 and a trapped oocyte undergoing cleavage (B) X 320.

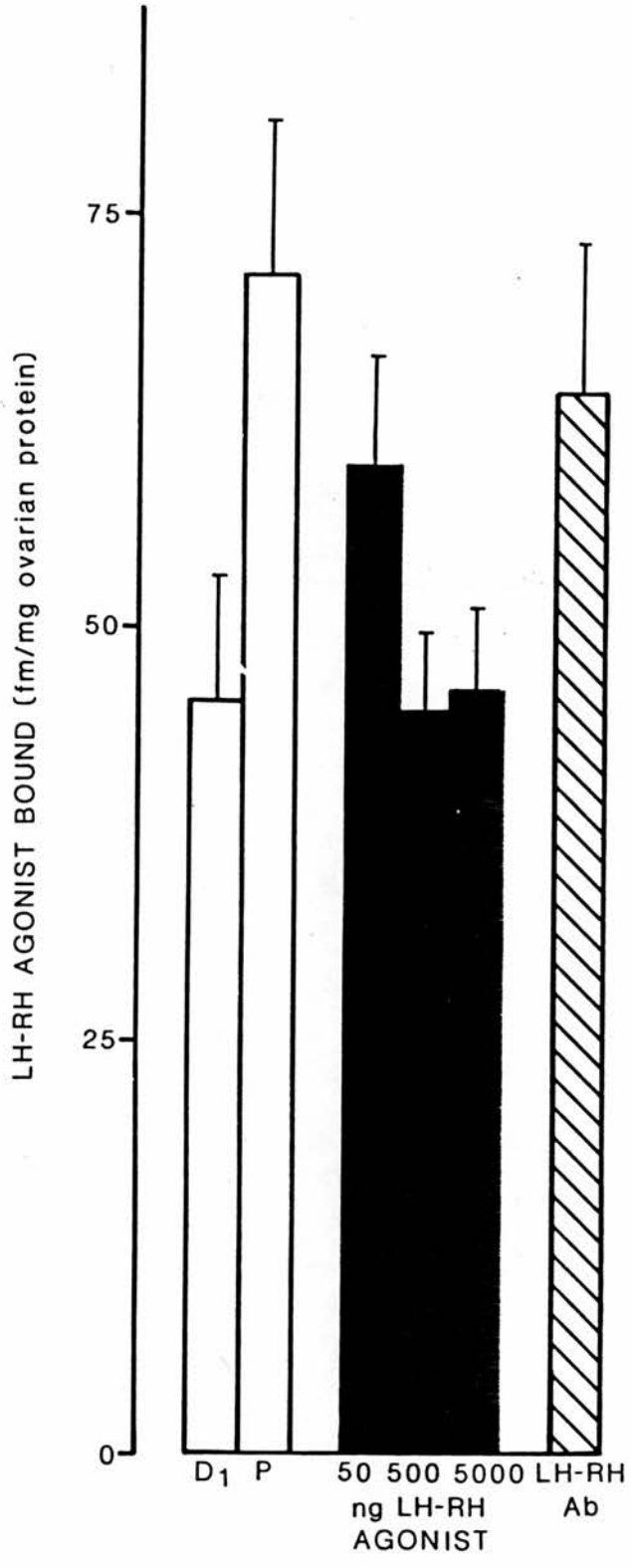

FIGURE 5.2 Effect of daily injections of 50, 500 or 5,000 ng LHRH agonist (■) or 3 times weekly injections of LHRH antibodies (▨) on ovarian LHRH receptors. Control animals received vehicle alone (□) and receptors were determined on the first day of dioestrus (D_1) or on pro-oestrus (P).

Values are Mean ± S.E.M. with 8 animals per group.

Ovarian LHRH receptor concentrations were not significantly different either between pro-oestrous and dioestrous control levels or after LHRH agonist treatment (Fig. 5.2). Results were not altered by expression of results as fm/ovary.

LHRH antiserum treatment: Immunoneutralization of LHRH also led to cessation of oestrous cycles, as evidenced by constant dioestrous type smears, decreased ovarian weight and serum oestradiol, as well as marked changes in ovarian morphology consistent with an impared ovulatory LH surge. There was no evidence of inhibition of follicular growth in antibody treated rats. Half the animals had no corpora lutea and contained large Graafian follicles with an abnormally enlarged antrum (i.e. cystic follicles). Ovarian LHRH receptors were not significantly different from control dioestrous values in treated animals (Fig. 5.2).

5.4.3 Discussion

Both LHRH agonist and LHRH antibody treatment resulted in marked but opposite changes in ovarian morphology. The former was associated with a predominance of luteal tissue (see also Sandow, 1982) and an inhibition of follicular growth (or an induction of atresia); the latter was consistent with impaired LH surges and resultant over mature and atretic follicles. Also present were cystic follicles similar to those seen in rats in persistent oestrus or when ovulation has been blocked by pentobarbitone (Everett & Sawyer, 1950; Braw and Tsafriri, 1980), or after active immunization against LHRH (Fraser & Baker, 1978). The contribution of possible direct inhibitory effects of LHRH agonist on follicular development is unknown.

The absence of an associated progesterone rise despite abundant luteal tissue in the LHRH agonist treated groups is interesting. This could be due to desensitization of the ovary after excess gonadotrophin stimulation (e.g. Conti et al., 1976) and/or direct inhibitory effects of LHRH (e.g. Clayton et al., 1979a). Since cystic follicles are usually associated with increased progesterone (Braw & Tsafriri, 1980) these structures may contribute to the serum progesterone concentrations after antibody treatment.

The absence of changes in ovarian LHRH receptor concentrations despite such marked changes in morphology and extremes of gonadotrophin exposure induced by LHRH agonist and antibody treatment, is surprising. This is likely to be due to the observation that LHRH binds to most cell types within the ovary (see autoradiographic data in Chapter 3) and would therefore be unlikely to be affected by changes in relative abundance of specific tissue types. Moreover, Pieper et al., (1981) reported that thrice daily injections of 6 μg LHRH to intact females failed to alter the number of ovarian receptors, a finding consistent with the above observations using LHRH agonist.

However, ovarian LHRH receptors have been reported to increase 6 and 24 hrs after a single injection of 10 μg LHRH, to PMSG-hCG primed immature rats (Harwood et al., 1980b) and 3 times daily injections of LHRH to rats with predominantly follicular or luteal tissue results in increased receptors (Pieper et al., 1981). This indicates that the effects of exogenously administered LHRH may depend on the predominant tissue present at the onset of treatment.

5.5 OVARIAN LHRH RECEPTORS AND MORPHOLOGY IN RATS ACTIVELY IMMUNIZED AGAINST LH OR LHRH

5.5.1 Method

Individual ovarian receptors were assessed in one ovary from each of 6 rats immunized against LH and 11 rats immunized against LHRH. Of 10 control rats immunized against HSA, 5 were killed at 10.00 h dioestrus and 5 at 12.00 h pro-oestrus, (see Chapter 4.4.3 for details). The remaining ovary in each case was taken for histology.

5.5.2 Results

Similar morphological changes were observed in ovaries of rats immunized against LH and LHRH, indicating inhibition of ovulation but not follicular development. Thus the ovaries were characterized by predominance of follicular tissue with an absence of luteal tissue (Plate 5.4). Interestingly, ovaries from 2 of the spontaneously non-cyclic control animals (which were excluded from the study - see Chapter 4.4.3) showed morphological characteristics similar to those seen in ovaries from rats exposed to constant light previously discussed in section 5.3. In addition the ovarian receptor concentrations were high (28 and 32 fm/mg protein) compared with the normal dioestrous control value (16 ± 3 fm/mg protein). Impairment of ovarian function was also indicated by a marked decrease in uterine and ovarian weight and constant dioestrous smear pattern.

Ovarian LHRH receptor concentrations were significantly greater than controls in both immunized groups ($P < 0.001$). If the data was expressed as fm bound/ovary however, no significant difference was

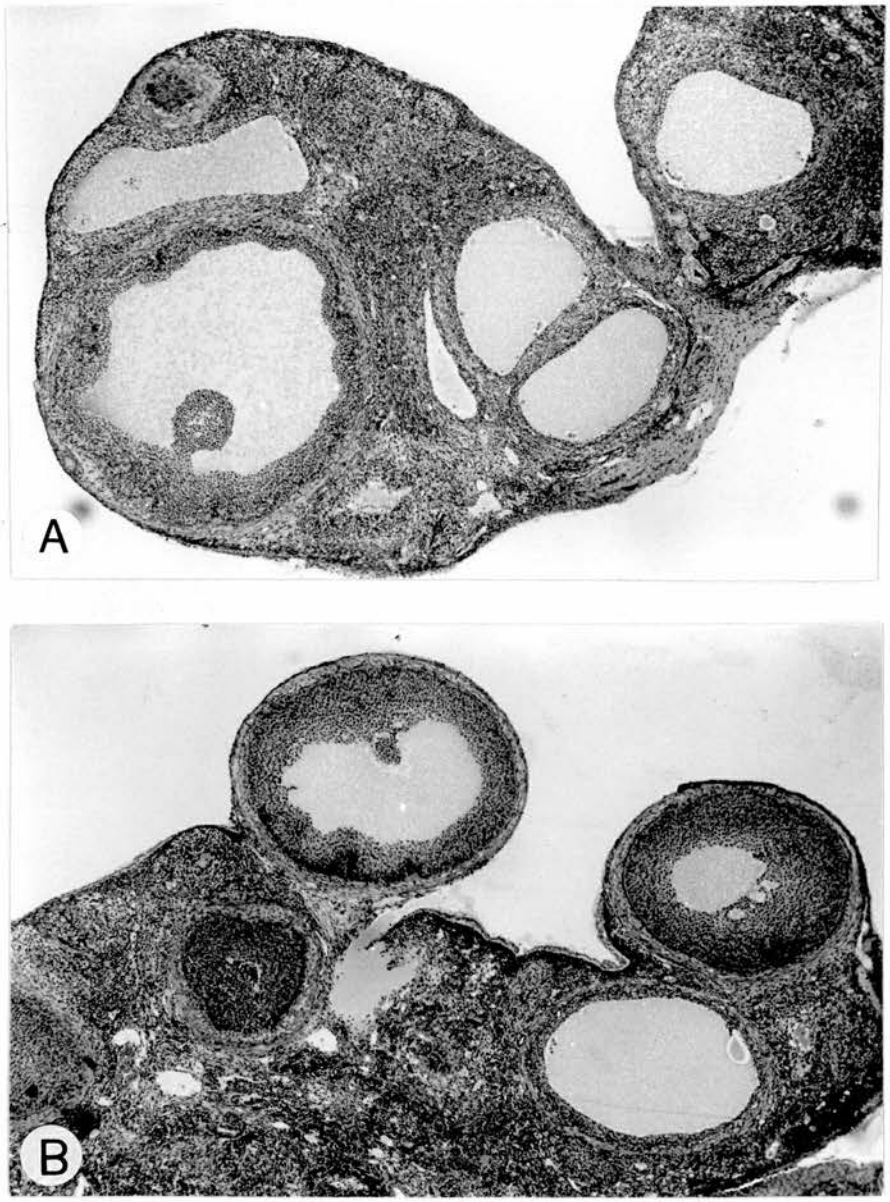

PLATE 5.4 Histological section through rat ovaries from rats immunized against LH (A) or LHRH (B). X 50.

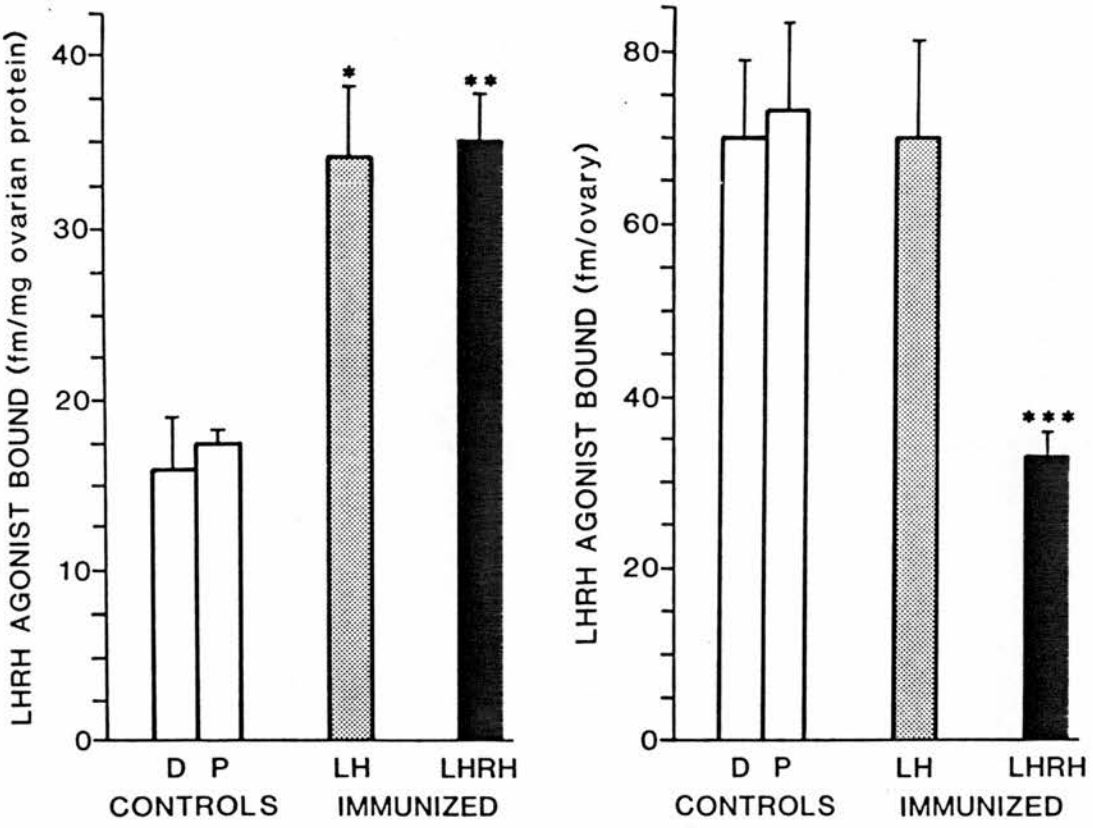

FIGURE 5.3 Ovarian LHRH receptors measured in control rats (□) at 10.00 h dioestrus (D) or 12.00 h pro-oestrus (P) and in rats actively immunized against LH (▨) or LHRH (■).

Data are expressed as fm bound per mg ovarian protein and as fm bound per ovary, and represent Mean + S.E.M. (7 rats in each control group, 11 and 6 in the LHRH and LH immunized groups respectively).

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ for immunized compared to control D value.

seen with LH immunization whereas immunization of LHRH resulted in a significant ($P < 0.001$) decrease in receptor numbers (Fig. 5.3).

This data contrasts with that of the pituitary where active immunization against LH increased receptor numbers, whereas immunization against LHRH, reduced receptor concentrations (Chapter 4.4.3).

5.5.3 Discussion

Ovarian LHRH receptor concentrations were markedly increased subsequent to active immunization against LH. This suggests that LH, and perhaps steroids, are negative regulators of receptor concentrations. Decreased receptor concentrations have been reported following treatment of immature rats with gonadotrophins (PMSG and hCG) (Harwood et al., 1980b), or following sequential treatment of immature hypophysectomized rats with FSH, LH and PRL (Jones et al., 1980).

The marked increase in receptor concentration following active immunization against LHRH is interesting, the reverse pattern being true for the pituitary (see Chapter 4.4.3). It could be suggested that either an intragonadal LHRH-like factor is immunoneutralized by the antisera, thus removing a negative regulator, or that the effects are due to a concurrent reduction in LH in LHRH-immunized rats. The latter explanation seems more probable, since it is unlikely that all immunized rats would develop antibodies cross reacting with a putative LHRH-like factor, in response to immunization against LHRH.

It is interesting to speculate on the significance of negative regulation of ovarian LHRH receptors by gonadotrophin. Since LHRH inhibited LH and FSH induced steroidogenesis (see Chapter 1) the

decrease in LHRH receptors with increased LH would enable full expression of gonadotrophin induced steroidogenesis. However, as will be discussed in Chapter 7, it appears that the short term effects of LHRH on basal events in follicular tissue are stimulatory. In this case in the presence of low LH, LHRH receptors would increase and thus ensure continuation of basal steroidogenesis.

Since follicular tissue has been reported to contain higher concentrations of LHRH receptors than luteal tissue (Pieper et al., 1981) it is interesting that the three treatment regimes reported to be associated with high ovarian receptor concentrations (namely, exposure to constant light or active immunization against LH or LHRH) are also associated with similar changes in ovarian morphology. Increased ovarian LHRH receptor concentrations were therefore correlated with the predominance of follicular tissue with a marked absence of luteal tissue.

5.6 OVARIAN LHRH RECEPTORS IN CYCLIC RATS PASSIVELY IMMUNIZED AGAINST LH OR LHRH

5.6.1 Method

Ovarian LHRH receptors were assessed in individual ovaries taken from rats killed at 12.00 h pro-oestrus (having been passively immunized against LH, LHRH or BSA at 10.00 h dioestrus day one) and from rats killed at 09.00 h oestrus (having been passively immunized against LH, LHRH or BSA at 12.00 h pro-oestrus - see Chapter 4.4.5 for further details).

5.6.2 Results

Passive immunization against LH or LHRH disrupted ovarian function as evidenced by presence of dioestrous type smears on

TABLE 5.1

Ovarian LHRH receptors (fm LHRH agonist bound/mg ovarian protein) at 11.00 h dioestrus (A) and 12.00 h pro-oestrus (B) and after passive immunization at 11.00 h dioestrus (A) and 12.00 h pro-oestrus (B) with LH or LHRH antisera.

Data are mean ± SEM 5 animals per group.

Stage of cycle	Control	Antiserum treated LH	LHRH
(A)			
11.00 h Dioestrus	21 ± 2		
12.00 h Pro-oestrus	24 ± 4	21 ± 3	25 ± 4
(B)			
12.00 h Pro-oestrus	25 ± 3		
09.00 h Oestrus	29 ± 3	24 ± 2	33 ± 4

the expected day of pro-oestrus in rats immunized on dioestrus one, and a failure of ovulation in rats immunized on pro-oestrus. Ovarian LHRH receptor concentrations were not significantly different either between control rats at dioestrus, pro-oestrus and oestrus or between control and treated rats at any time (Table 5.1). No difference was detected if results were expressed as fm LHRH bound per ovary.

5.6.3 Discussion

Short-term passive immunoneutralization of LH or LHRH failed to alter ovarian LHRH receptor concentrations despite both treatments abolishing ovulation and reproductive function. This contrasts with data on pituitary LHRH receptors presented in Chapter 4.4.5 showing decreased receptors, after LHRH immunoneutralization, at pro-oestrus.

The data suggest that LH, steroids and LHRH are not involved in the short-term regulation of ovarian LHRH receptors. This is in contrast to the situation in the male, where passive immunization against LH resulted in increased testicular LHRH receptors (Sharpe & Fraser, 1983). Since prolonged deprivation of LH or gonadotrophin stimulus by active immunization against LH or LHRH resulted in increased ovarian LHRH receptor concentrations (see 5.5), whereas short-term immunoneutralization had no effect, it is likely that gonadotrophins are involved in regulating the long-term, but not short-term, ovarian LHRH receptor concentration.

5.7 CONCLUSIONS

The experiments described in Sections 5.3 - 5.6 are summarised in Table 5.2 and are consistent with the following conclusions concerning the regulation of ovarian LHRH receptor concentrations:-

TABLE 5.2

Summary of changes in pituitary and ovarian LHRH receptors during the experiments described in Chapters 4 and 5.

Arrows indicate known and assumed direction of change relative to controls.

Treatment	Pituitary Receptors	Ovarian Receptor Concentrations	Serum LH	Steroids	Uterine Wt.	Ovarian Wt.
LHRH agonist - 3 weeks.	-	-	-	↓	↓	↓
LHRH antiserum - 3 weeks.	-	-	↑	↑	-	↑
Active Immunization LH	↑	↑↑	↑↑↑	↑↑	↑↑	↑
Active Immunization LHRH	↓↓	↑↑	↑↑	↑↑	↑↑	↑
Passive Immunization LH	-	-	↑↑	↑	↑	-
Passive Immunization LHRH	↓	-	↑	↑	↑	-
Constant light	-	↓	-	-	-	↑

(a) Ovarian and pituitary LHRH receptors are not concomitantly controlled.

(b) Hyperstimulation with LHRH agonist had no effect on ovarian LHRH receptor concentrations.

(c) Gonadotrophins are likely to be involved in the long-term, but not the short-term regulation of ovarian LHRH receptor concentrations.

5.8 GENERAL DISCUSSION

The above conclusions were based on data obtained from analyses of LHRH receptor concentrations in total ovarian homogenates. There are several problems with this model, which are revealed by a consideration of the nature and characteristics of ovarian LHRH binding. Firstly, binding sites for LHRH are present in most cell types within the ovary (e.g. granulosa, theca and luteal tissue). Binding is diffuse, not localized on specific clusters of cells as in the pituitary (see autoradiographic data in Chapter 3). Therefore analysis of total receptor concentration does not enable differences in receptor numbers between compartments to be determined. Secondly, the complexity of LHRH action on ovarian tissue (see Chapters 1 and 7) means that there are no well-defined data available on sensitivity of the different cell types to LHRH, so the physiological significance of changes in receptor concentrations remains unsolved. Finally the identity, source and pattern of secretion of the putative endogenous ovarian LHRH-like ligand is unknown. This contrasts with the pituitary where detailed evidence is available as to the sensitivity and pattern of secretion of LHRH (see Chapter 4).

LHRH receptor concentrations are likely to be regulated within

different ovarian compartments. Separate analyses of receptor concentrations revealed higher levels in granulosa cells than in luteal and residual ovarian tissue (Pieper et al., 1981). Studies utilizing isolated granulosa cells in vitro have shown that receptors are biphasically regulated by LHRH agonist, being increased in the presence of low doses and decreased in the presence of higher levels (Ranta et al., 1982). In addition granulosa cells cultured in the absence of FSH showed decreased receptor numbers (Ranta et al., 1982). Confirmation that granulosa cell LHRH receptors are indeed regulated in vivo has come from data on cells isolated from pre-pubertal rats. The loss of binding prior to the first LH surge (Smith-White & Ojeda, 1981) was reflected by loss of granulosa cell, but not residual thecal/interstitial cell, LHRH receptors (Smith-White & Ojeda, 1983).

Studies in the male are simplified by the fact that only one cell type within the testis, namely the Leydig cells, possesses LHRH receptors and essentially one biological response, namely testosterone production, is influenced by LHRH (see Sharpe et al., 1982a for review). In the male, hypophysectomy resulted in increased testicular LHRH receptors (Bourne et al., 1980) and exogenous administration of LHRH further enhanced binding capacity (Bourne et al., 1980). However in the female neither hypophysectomy nor subsequent LHRH administration had any effect on ovarian receptors (Pieper et al., 1981). Testicular LHRH receptors were increased after injection of LHRH to intact rats and this increase was associated with an enhanced response to LHRH (Bourne et al., 1980). In contrast, treatment with LHRH agonist was reported to decrease testicular LHRH receptors (Sharpe & Fraser, 1980a).

Heterologous regulation of testicular LHRH receptors by LH has been suggested by the observations that hypophysectomy (Bourne et al., 1980) or passive immunization against LH (Sharpe & Fraser, 1983) increases both Leydig cell LHRH receptors and increases Leydig cell reponsiveness to LHRH (Sharpe & Fraser, 1983). *

Thus, testicular LHRH receptors, and to a limited extent isolated granulosa cells, appear to be regulated in a manner analogous to that of pituitary LHRH receptors, showing evidence for both heterologous regulation by LH and homologous regulation by LHRH. However, the regulation of ovarian receptors in other ovarian compartments remains largely unsolved. Perhaps follicles are only affected by LHRH as they pass through a certain stage of development and after active immunization the effects of prolonged deprivation of LH are revealed. Elucidation of the significance of ovarian LHRH receptor changes and ovarian responsiveness to LHRH must await detailed studies of receptor concentrations and steroidogenic effects within specific ovarian compartments.

* Ovarian LHRH receptors have been reported to decrease 24hr after administration of hCG suggesting that hCG is capable of exerting a short-term effect on ovarian LHRH receptor numbers (Harwood et al. 1980b).

CHAPTER 6

BINDING OF LHRH TO THE HUMAN CORPUS LUTEUM

CHAPTER 6

BINDING OF LHRH TO THE HUMAN CORPUS LUTEUM

6.1 INTRODUCTION

6.2 SOURCE OF TISSUE

6.3 ASSESSMENT OF LHRH AGONIST BINDING

6.4 TIME COURSE OF LHRH AGONIST BINDING

6.5 SEPARATION OF FREE FROM BOUND

6.6 SPECIFICITY OF BINDING

6.7 DISCUSSION AND CONCLUSIONS

6.1 INTRODUCTION

Direct gonadal actions of LHRH have been demonstrated in a variety of species e.g. rat (see Chapter 7; e.g. Clayton et al., 1979a), pig (Massicotte et al., 1980), rabbit (Koos et al., 1982), cow (Milvae & Hansel, 1980) and chicken (Hertelendy et al., 1982). These extra-pituitary actions are likely to be mediated via specific LHRH receptors present on ovarian and testicular tissue (Chapter 3; Sharpe, 1982; Ranta et al., 1982). However, the relevance of these observations to the human remains uncertain. Since LHRH and its analogues are used clinically to modify reproductive function (see Chapter 1) it is clearly of primary importance to determine whether LHRH is capable of acting at sites other than the pituitary in the human.

6.2 SOURCE OF TISSUE

Human luteal tissue was obtained from women undergoing laparotomy during the luteal phase of the cycle and human postmenopausal tissue was obtained from women undergoing hysterectomy (the informed consent of the patient was obtained for this procedure). The tissue was provided by Dr. R.W. Shaw and Prof. D.T. Baird from the Royal Infirmary, Edinburgh.

The tissue was transported immediately after removal from the patient to the laboratory where it was either stored at -70°C or -40°C, or used directly for the binding assay.

Hormones: LHRH agonist$_1$) Kindly provided by
(D-Ser but^6 des Gly^{10} LHRH ethylamide)) Dr. J. Sandow
TRH) (Hoechst, U.K.)
Somatostatin)
LHRH agonist$_2$ (6-D-2-naphthyl-alanine-LHRH), D-Nal (2)^6
LHRH was provided by Dr. B. Vickery (Syntex)

Oxytocin was a gift from Sandoz labs.

6.3 ASSESSMENT OF LHRH AGONIST BINDING

Ovarian tissue was homogenized in 10 mM Tris HCl (0.3 M sucrose, 1 mM EDTA pH 7.4) using either a Polytron homogenizer or a glass pestle hand homogenizer. Aliquots of homogenate were then used for the binding assay. Incubations were carried out in duplicate or triplicate in the presence of ^{125}I-LHRH agonist (100,000 - 120,000 cpm) and non-specific binding (NSB) was determined in the presence of 10^{-5} M unlabelled hormone.

These studies were performed in collaboration with Dr. T.A. Bramley.

6.4 TIME COURSE OF LHRH AGONIST BINDING

Initial studies were performed utilizing the methods described for analysis of rat LHRH receptors (Chapter 3). The incubation was set up as described above for various times on ice, with separation of free from bound being achieved by filtration under vacuum through Whatman GF/C glass fibre filters, pre-soaked in 2% BSA, followed by washing with 3 x 2 ml PBS. The results obtained for luteal tissue and post-menopausal ovarian tissue are shown in Fig. 6.1. Luteal tissue specifically bound ^{125}I-LHRH agonist (4.5% of total counts added) whereas no binding could be detected to post-menopausal ovarian tissue. Maximum binding to luteal tissue was obtained after 120' incubation at 0-4°C.

In a separate study luteal homogenates were incubated for different times and temperatures to confirm the point of maximum binding (Fig. 6.2). Again binding was shown to be time and temperature dependent with rapid binding following by a sharp decline at 37°C (presumably due to rapid ligand and/or receptor degradation at this temperature) and a slower rise to maximum levels at 20° and 0-4°C. Binding assays were therefore routinely carried out for either 1 hr at 20°C or 2 hrs at 0-4°C.

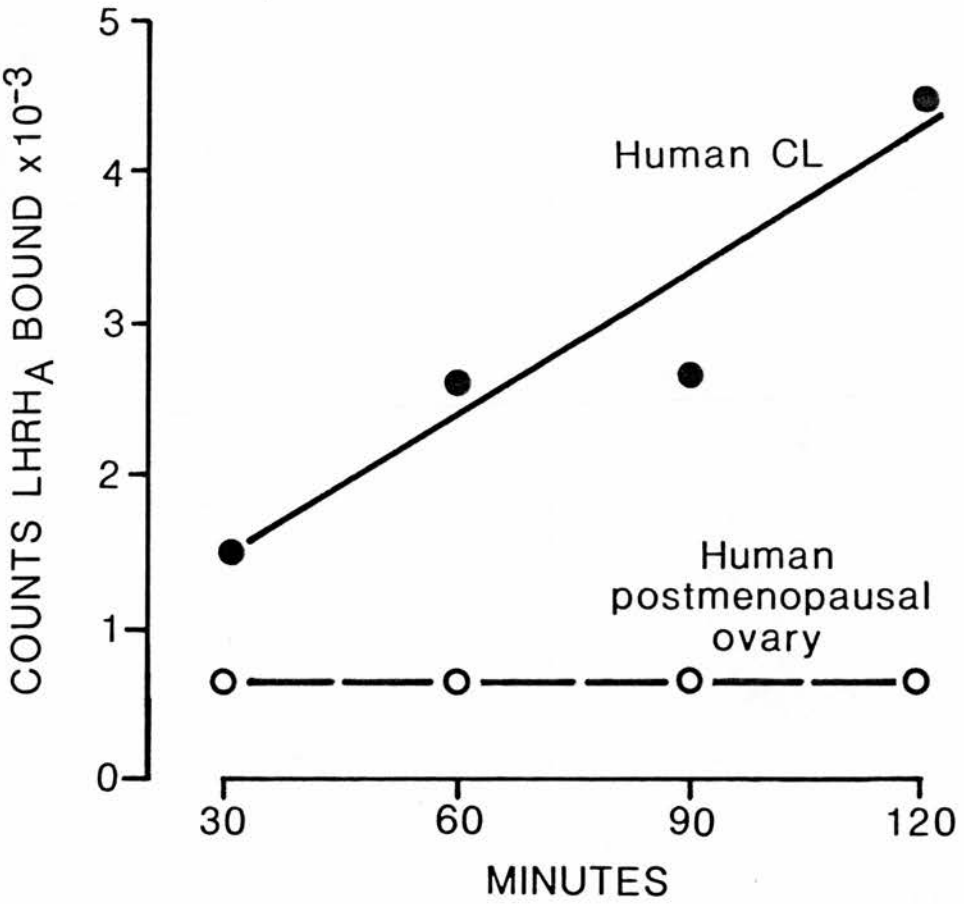

FIGURE 6.1 Specific binding of ^{125}I-LHRH agonist (LHRH$_A$) to human corpus luteum tissue and to human postmenopausal ovarian tissue. Each point represents mean of duplicate estimations. Replication of duplicates was within X 15% of the mean values.

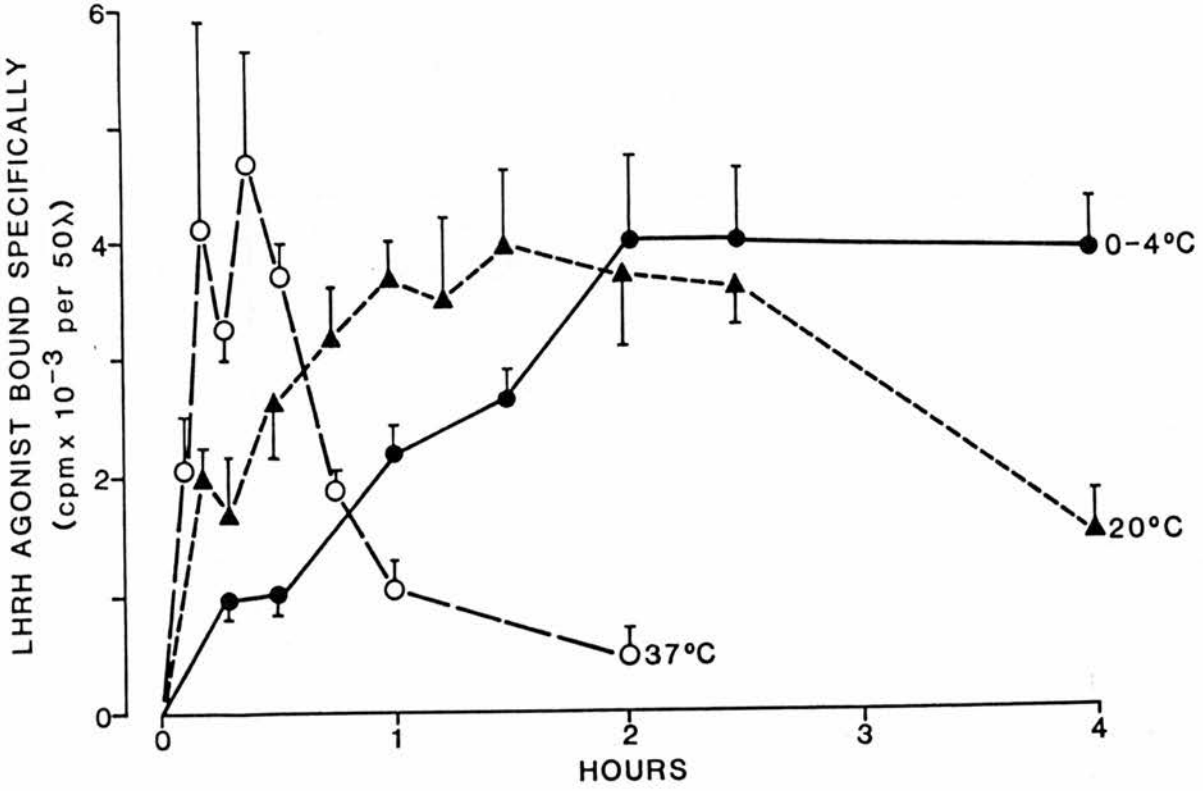

FIGURE 6.2 Specific binding of ^{125}I-LHRH agonist to human corpus luteum homogenates at different temperatures for periods up to 4 h. The patterns of specific binding with time and temperature were similar in 3 separate experiments using different luteal homogenates. Hence the data have been combined (after normalization to a plateau value of 4,000 pg/50 µl to correct for differences in homogenate amounts). Each point represents mean specific binding ± ranges.

6.5 SEPARATION OF FREE FROM BOUND

Several methods of separating free from bound hormone were examined either based on filtration using various Millipore filters or polyethylene glycol (PEG) precipitation (as described in Chapter 3). A number of Millipore filters were tested. Duropore disc filters composed of a polymer of polyvinylidine fluoride (PVF) with pore sizes 0.22 μ (GVWP) or 0.45 μ (HVLP), mixed ester filters composed of cellulose acetate (CA) and cellulose nitrate (CN) with pore size 0.45 μ (HAWP), celotate filters composed of pure inert CA and pore size 0.5 μ (EHWP) and finally a bonded acylate pre-filter (GF). All filters were pre-soaked in 2% BSA to minimize non specific adsorption of tracer.

Specific binding of LHRH agonist to human luteal tissue could only be detected by EHWP, GVWP, Whatman GF/C filter and PEG precipitation (Fig. 6.3), the latter method giving the greatest degree of recovery of bound hormone.

The binding assay was therefore routinely terminated by chilling the tubes on ice before the addition of 0.5 ml 0.5% bovine immunoglobulin and 1 ml 25% PEG followed by thorough vortexing and centrifugation at 2000 rpm for 15 minutes at 4°C. Radioactivity retained in the pellet was then measured in a gamma counter.

During the course of these studies a report was published failing to demonstrate the presence of specific binding of ^{125}I-LHRH agonist to human luteal tissue (Clayton & Huhtaniemi, 1982). Using this method, namely incubation in the presence of 10,000 cpm ^{125}I-LHRH agonist and an NSB of 10^{-8} M LHRH agonist with separation by Whatman GF/C filters, no specific binding was detected (Fig. 6.3).

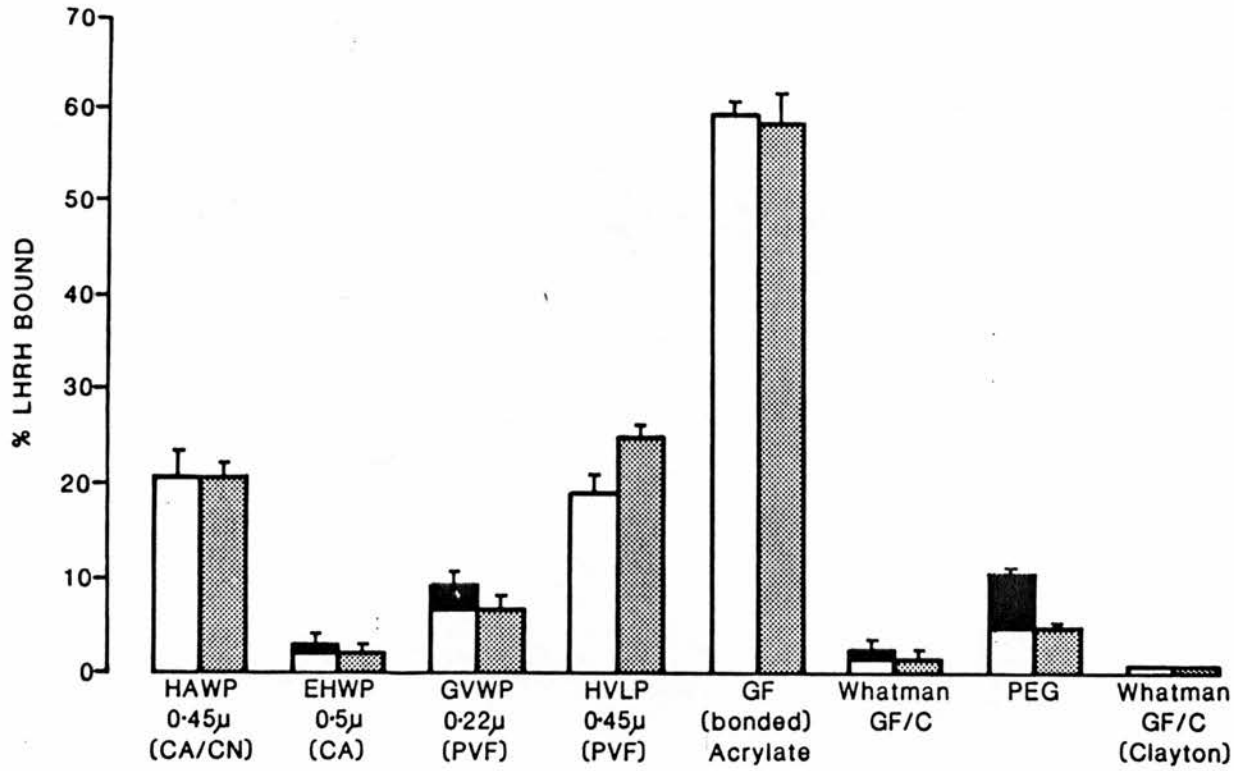

FIGURE 6.3 % Binding of ^{125}I-LHRH agonist (LHRH) to human corpus luteum homogenates with non specific binding (▨), total binding (□) and specific binding (■) after separation of free from bound using various Millipore filters (see text for details) or PEG precipitation. Bars represent mean ± range for duplicate determinations.

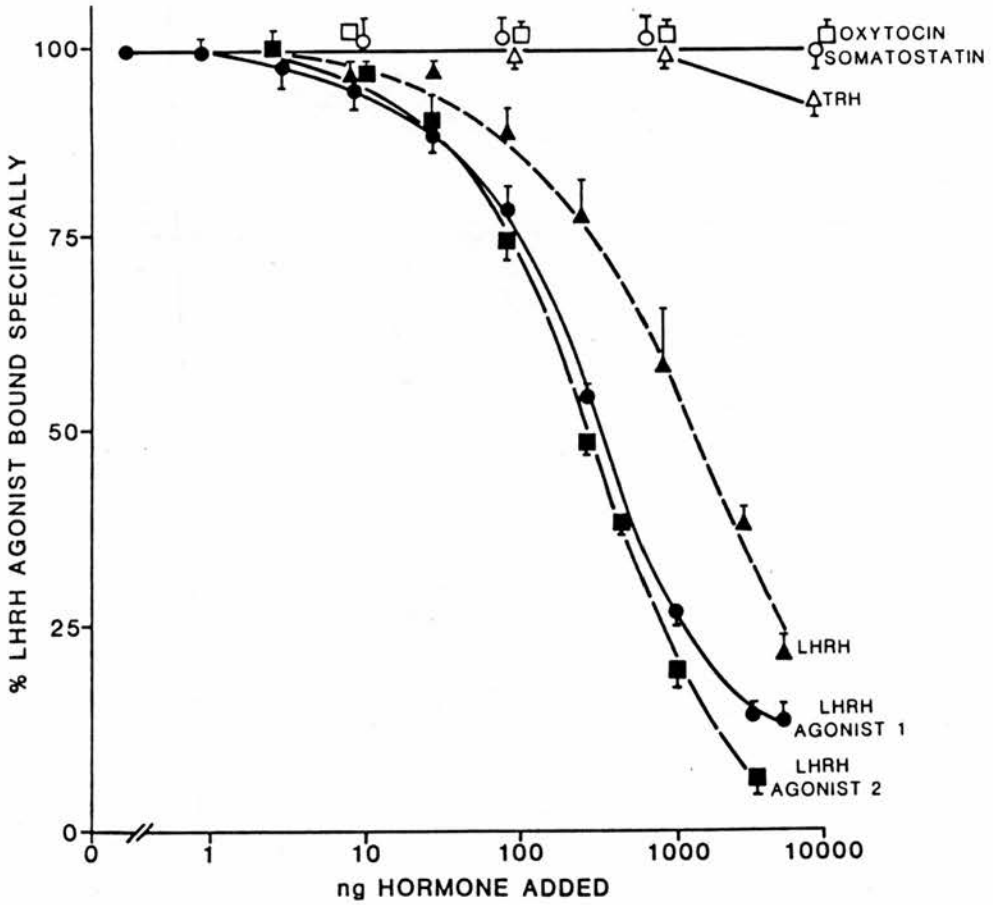

FIGURE 6.4 Displacement of binding of ^{125}I-LHRH agonist 1 by (D-Ser-t-Bu^{6}-des Gly NH$_2^{10}$) LHRH ethylamide, LHRH agonist 2 (D-Nal (2)^{6} LHRH), LHRH, oxytocin, somatostatin and TRH. The figure represents the mean ± ranges of 3 displacement curves from 3 human corpora lutea.

6.6 SPECIFICITY OF BINDING

The specificity and affinity of the binding sites for LHRH were examined by testing the ability of unlabelled hormones to compete for binding of the iodinated agonist (Fig. 6.4). The apparent affinity constants (Ka) calculated from the displacement curves with LHRH agonist as tracer, were found to be 3×10^7 M^{-1} in 3 separate experiments using different corpus luteum homogenates (Fig. 6.4). This value was also obtained for a second LHRH agonist ((D-Nal (2)^6) LHRH). Native LHRH gave an apparent Ka of 10^6 M^{-1} indicating a slightly lower affinity of the binding site for LHRH. Binding was specific for LHRH and LHRH agonists, neither oxytocin, somatostatin nor TRH competing for binding at concentrations up to 10,000 ng.

6.7 DISCUSSION AND CONCLUSIONS

These results demonstrate that binding sites for LHRH are present on human luteal tissue and that these sites are specific for LHRH and related peptides. The studies indicated that for native LHRH, the apparent affinity of binding to the human corpus luteum was similar to the value reported for binding to the rat pituitary (Clayton et al., 1979b) and gonadal tissue (Clayton & Catt, 1981b; Chapter 3). In marked contrast, human ovarian binding sites had similar affinities for the two agonists and for native LHRH, indicating a clear species difference.

These findings are in contrast to a recent report in which LHRH binding to human luteal tissue was not detectable (Clayton & Huhtaniemi, 1982). The reasons for this discrepancy became clear from the data presented here on the nature of the binding site. In order to detect the presence of a relatively low affinity receptor, it is necessary to use both a sufficiently high concentration of radiolabelled ligand and a

sufficiently high concentration of unlabelled hormone (10^{-5}-10^{-6} M) to assess non-specific binding. Using the method of Clayton and Huhtaniemi (1982) with a lower concentration of both unlabelled (10^{-8}M) and labelled (10-50,000 cpm) ligand, no binding was detected to a homogenate of human luteal tissue. The same tissue in the assay described, specifically bound 37.5 pg LHRH agonist/µg DNA.

Several possible explanations for the low apparent affinity of LHRH binding to the human corpus luteum can be suggested. Firstly, the apparent Ka ($3 \times 10^{7}M^{-1}$) may represent the true affinity of the binding site, in which case its physiological significance remains uncertain due to the relatively high concentrations which would be necessary to activate the receptor. Secondly, the true affinity of the receptor may be greater than $10^{7}M^{-1}$, but the agonist and native hormone may be degraded to a similar extent (as suggested for human placental tissue (Currie et al., 1981)) giving rise to apparent low-affinity binding. Thirdly, the putative intragonadal factor(s) interacting with such a binding site in vivo are unlikely to be identical to hypothalamic LHRH or its agonist. Indeed, LHRH-like molecules detected in rat testicular tissue have been shown to be non-identical to LHRH (Dutlow & Millar, 1981; Sharpe et al., 1981). Thus the physiological ligand for such receptors may well have a greater affinity than either LHRH or LHRH agonist. In addition there is no data available on the affinity of the human pituitary LHRH receptor (and indeed the levels of specific binding reported were extremely low in comparison to rat tissue (0.9 - 2.3% of total counts specifically bound for human pituitaries, compared with 16-26% for rat - see Clayton & Huhtaniemi 1982). It is also possible, but unlikely, that human pituitary LHRH receptors have different

affinities from those of rat.

In a similar study, but utilizing ovarian tissue from rhesus monkeys, no specific binding of LHRH agonist was detected (Asch et al., 1981). However, although a high NSB (10^{-4}M) was utilized, the total number of counts added was low (10,000 cpm). It would be of interest to repeat such studies using a greater quantity of ^{125}I-LHRH agonist.

The possibility that human ovarian binding sites might mediate direct effects of LHRH has been suggested by the observation that isolated human granulosa cells, when incubated for prolonged periods (96 h) with LHRH agonist, showed a 65% reduction in basal progesterone production (Tureck et al., 1982). However, a similar study failed to show any effect of native LHRH on basal or FSH-induced steroidogenesis in isolated human granulosa cells (Casper et al., 1982). Neither was any effect seen on progesterone production from human (Tan & Biggs, 1983) or rhesus monkey (Asch et al., 1980) luteal cell progesterone production, although a recent report implicated a direct effect of LHRH on progesterone production from cultured monkey granulosa cells (Knecht et al., 1983).

The possibility of direct testicular effects of LHRH has not been examined in the human. LHRH suppression of basal testosterone from an isolated Sertoli-Leydig cell tumour has been reported (Lamberts et al., 1982). Thus LHRH may well bind to human testicular tissue. Preliminary data suggests that isolated human Leydig cells are capable of specific binding of ^{125}I-LHRH agonist with similar displacement characteristics to those shown for human luteal tissue (Sharpe - personal communication).

Extra hypothalamic LHRH-like material has been detected in the human in carcinoid tumours (Wahlström & Seppälä, 1981), milk (Amarant et al., 1982), peritoneal fluid (Demoulin et al., 1981) and placenta (Khodr &

Siler-Khodr, 1978b & 1980; Seppälä et al., 1980). However, as will be discussed in Chapter 8, the detection of such material is subject to many pitfalls. The physiological significance, if any, of both human LHRH-like molecules and LHRH binding sites in the endogenous control of ovarian function, remains to be determined. It seems likely that the discovery of these binding sites owes a great deal to serendipity and that the true ligand has a higher affinity for the binding sites.

However, since human luteal binding sites have such a low affinity for LHRH and LHRH agonist, it is highly unlikely that even large clinically administered doses would induce circulating concentrations high enough to interact with the binding sites. Thus circulating concentrations greater than 0.3 μg/ml of LHRH agonist or 1 μg/ml for LHRH would need to be achieved to reach the Ka for the binding site, requiring i/v administered doses in excess of 1.4 mg LHRH agonist or 45 mg LHRH.

Thus, from a clinical point of view, small doses administered over prolonged periods of time are unlikely to interact with these binding sites whereas huge doses administered at specific times may activate ovarian binding sites. It seems likely therefore, that the antifertility effects of LHRH agonists in the human discussed in Chapter 1 are mediated through changes in pituitary gonadotrophin release rather than direct gonadal effects.

With new techniques available for the isolation of rat pituitary and ovarian LHRH receptors (see Chapter 3) it is hoped that the application of this technology to human luteal tissue will resolve whether the binding demonstrated is physiologically relevant or represents binding to a low-affinity protein such as a protease.

CONCLUSIONS

1. The human corpus luteum possesses binding sites for LHRH and LHRH agonist.
2. These binding sites are absent from human post-menopausal ovarian tissue.
3. The binding sites are specific for LHRH and LHRH agonist but have a low affinity for LHRH agonist.
4. The nature of the binding site (receptor for endogenous ligand or enzyme) is unknown.
5. Circulating levels of LHRH or LHRH agonist induced by exogenous administration for clinical purposes are unlikely to be high enough to interact with such sites *in vivo*.

CHAPTER 7

DIRECT EFFECTS OF LHRH AND LHRH AGONIST ON STEROIDOGENESIS IN ISOLATED RAT FOLLICLES.

CHAPTER 7

DIRECT EFFECTS OF LHRH AND LHRH AGONIST ON STEROIDOGENESIS IN ISOLATED RAT FOLLICLES.

7.7 EFFECTS OF LHRH AGONIST ON hCG-INDUCED STEROIDOGENESIS

7.7.1 Effect on hCG dose response
7.7.2 Effect on time course of hCG action
7.7.3 Effect of pre-incubation
7.7.4 Summary

7.8 EFFECT OF LHRH AGONIST ON cAMP-INDUCED STEROIDOGENESIS

7.8.1 Effect on dibutyryl cAMP-induced changes
7.8.2 Effect on caffeine-induced changes

7.9 EFFECT OF LHRH AGONIST ON PROSTAGLANDIN-STIMULATED STEROIDOGENESIS

7.10 EFFECT OF PROSTAGLANDIN SYNTHESIS INHIBITORS ON LHRH AGONIST-STIMULATED STEROIDOGENESIS

7.10.1 Effect of indomethacin
7.10.2 Effect of aspirin

7.11 SUMMARY

7.12 RESPONSIVENESS OF FOLLICLES TO LHRH AGONIST DURING DIFFERENT STAGES OF DEVELOPMENT

7.12.1 "Immature" follicles
7.12.2 Dioestrous follicles

7.13 GENERAL DISCUSSION

7.14 GENERAL CONCLUSIONS

7.1 INTRODUCTION AND AIMS

The concept of direct ovarian actions of LHRH and LHRH agonist is now widely acknowledged for the rat, in the light of specific high affinity ovarian LHRH receptors (discussed in Chapters 3 & 4) together with direct effects on several parameters of ovarian function (discussed in Chapter 1.4.2.1). The direct actions reported appear to be predominantly inhibitory in nature, reducing gonadotrophin-induced events (see Chapter 1). However, several factors were noted from these studies. Firstly, the majority of results had been obtained utilizing prolonged incubation conditions ($>$ 48 hrs) and were concerned with gonadotrophin-induced events (see Hsueh & Jones, 1981 for review). Moreover few reports included data on 'control' incubations with LHRH effects on basal steroidogenesis (e.g. Hsueh & Erickson, 1979a). It was possible therefore that, as in the pituitary, the physiological response to short-term exposure to LHRH might be stimulatory, particularly to basal events.

Secondly the studies were largely confined to a consideration of granulosa cell function (as reflected by oestrogen and progesterone production) with only one report indicating a possible thecal/intersititial action (Magoffin et al., 1981). Thus an inhibition of androsterone production was noted after 4 day culture of collagenase-dispersed cells from immature rat ovaries (Magoffin et al., 1981). Although androgen release was measured, its source (i.e. thecal or interstitial cell) was not determined.

Since thecal androgen provides substrate and regulatory steroids essential for granulosa cell function (Leung & Armstrong, 1980; Hillensjö, 1981 for reviews) any actions of LHRH on thecal cells would clearly be of

primary importance in the regulation of follicular function. A model system was therefore sought which would enable assessment of thecal and granulosa cell function.

Isolated rat follicles:

Individual follicles can be isolated from rat ovaries and have been utilized to study, *in vitro*, the factors influencing follicular function. The first studies reported utilized Graafian follicles isolated from pro-oestrous rats (e.g. Tsafriri *et al.*, 1972; Tsafriri *et al.*, 1973; Lindner *et al.*, 1974). However, the use of follicles isolated from immature rats treated with pregnant mare's serum gonadotrophin (PMSG) to induce follicular development had the advantage of convenience and the production of a single crop of follicles of known age. Initial *in vitro* studies, examining LH responsiveness and oocyte maturation, indicated that follicles isolated from pro-oestrous rats or from rats killed 48 hrs after PMSG treatment, had esentially the same steroidogenic capacities (Hillensjö *et al.*, 1976). A dose of 4 international units (IU) PMSG was found to induce adequate follicular development of 10-12 follicles (after 48 h), oestrogen production, an LH surge and progesterone increase after 54 h, and a normal quota of ova 72 h later (e.g. Guillet & Rennels, 1964; Armstrong *et al.*, 1969; Armstrong *et al.*, 1976). Since this regime of PMSG treatment thus mimicked events of the oestrous cycle and had been utilized by a number of groups (e.g. Fortune & Armstrong, 1977 & 1978) it was adopted for the studies described in this Chapter.

AIMS

The following studies utilized isolated rat preovulatory follicles to examine:-

(1) The nature of the short-term effects of LHRH and LHRH agonist on basal steroidogenesis.

(2) The specificity, dose dependence and time course of such effects.

(3) Whether LHRH directly influences thecal function.

(4) The nature of short-term effects of LHRH agonist on gonadotrophin-stimulated events.

(5) The effects of LHRH agonist on prostaglandin- and dibutyryl cyclic AMP (db cAMP)-stimulated steroidogenesis.

(6) The effects of indomethacin and aspirin on LHRH agonist actions.

(7) Whether follicles from different stages of development respond to LHRH agonist.

7.2 MATERIALS AND METHODS

The following procedures were routinely adopted for the experiments described in this Chapter.

Animals: Immature female Sprague Dawley rats from the laboratory colony, housed under standard conditions (with lights on between 05.00 h - 19.00 h and access to food and water ad libitum), were injected subcutaneously (s.c.) with 4 IU PMSG between 8.30 - 10 a.m. on days 26 - 30 of age. 48 h after injection the animals were killed with dry ice CO_2 and the ovaries, trimmed of fat and oviduct tissue, removed to incubation medium.

Chemicals and Hormones:-

Incubation Medium (M199):-

Medium 199 containing 20 mM Hepes (Flow Labs) to which was added:-

2 mM Glutamine (Sigma)

0.35 g/l sodium bicarbonate

100 IU Penicillin) Difco Labs, Detroit

100 µg/ml Streptomycin) Michigan, U.S.A.,

0.2% BSA

Additions:-

LHRH agonist (D-Ser (Bu^t)^6 des Gly^{10} LHRH ethylamide)	)	Hoechst U.K.,
Thyrotropin releasing hormone (TRH)	)	
Growth hormone inhibiting hormone (GHIH)	)	
LHRH antagonist ($DPhe^2$ Phe^3 $DPhe^6$ LHRH)		gift from Dr. J. Sandow
Pregnant Mare's Serum Gonadotrophin (PMSG)		Folligon, Intervet, Cambridge
Human Chorionic gonadotrophin (hCG)		Chorulon, Intervet, Cambridge
Dibutyryl cyclic AMP (db cAMP))		
Caffeine	)	
*Indomethacin	)	(Sigma Chemicals)
Aspirin	)	
*Prostaglandin $F_2\alpha$ ($PGF_2\alpha$))		
*Prostaglandin E_2 (PGE_2)	)	

*Were added to the incubation medium in ethanol. In each case an equal volume of ethanol alone was added to control wells. No deleterious effect was seen on follicular steroidogenesis provided the ethanol concentration was $\lesssim$ 2%.

Isolation of follicles:- The largest follicles (5-6 per ovary 0.9 - 1.3 mm diameter) from each ovary were dissected out and trimmed of surrounding interstitial tissue under a stereomicroscope using fine dissecting

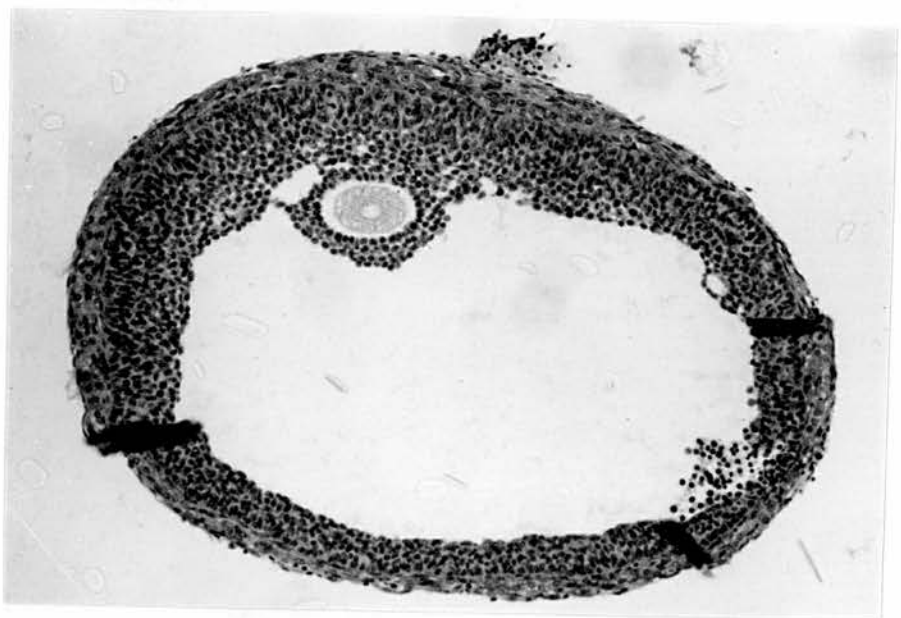

PLATE 7.1 Histological section through a single follicle isolated from the ovary of an immature rat 48 h after treatment with PMSG. X 50.

forceps (Plate 7.1). Follicles from each animal were pooled and 1-2 follicles from each animal were placed in each of the treatment groups. Each group contained 10 follicles which were incubated individually in Linbro multiwell plates in a final incubation volume of 0.5 ml. Incubations were carried out for various times in a modular incubation chamber gassed with 95% O_2/5% CO_2 at 37°C.

Steroid assays:- Steroids were assayed in duplicate from unextracted culture medium as described in Chapter 2, and the results expressed as pg or ng per follicle i.e. per 0.5 ml incubation medium.

Analysis of data:- Data presented represent mean ± S.E.M. of steroid values from 10 individually incubated follicles. Each figure shows the results from one representative experiment, each experiment having been repeated at least once.

Statistical analysis was assessed by Student's t test and/or analysis of variance.

7.3 DIRECT EFFECTS OF LHRH AND LHRH AGONIST ON BASAL STEROIDOGENESIS

7.3.1 Effect of LHRH Agonist on basal steroid release

Method:- Three groups of 20 follicles were incubated with or without 10^{-8} M LHRH agonist for 3 h, 6 h or 18 h, after which the medium was removed and stored at -20°C for steroid assay.

Results:- LHRH agonist caused significant increases ($P < 0.001$) in both androstenedione and testosterone release at 3 h, 6 h and 18 h (Fig. 7.1). Progesterone levels were non-detectable (< 50 pg/follicle) at 3 h and 6 h but a small but significant ($P < 0.02$) increase in progesterone was seen at 18 h (Control 332 ± 51 pg/follicle; LHRH agonist treated 913 ± 195 pg/follicle mean ± S.E.M.). Oestradiol release was

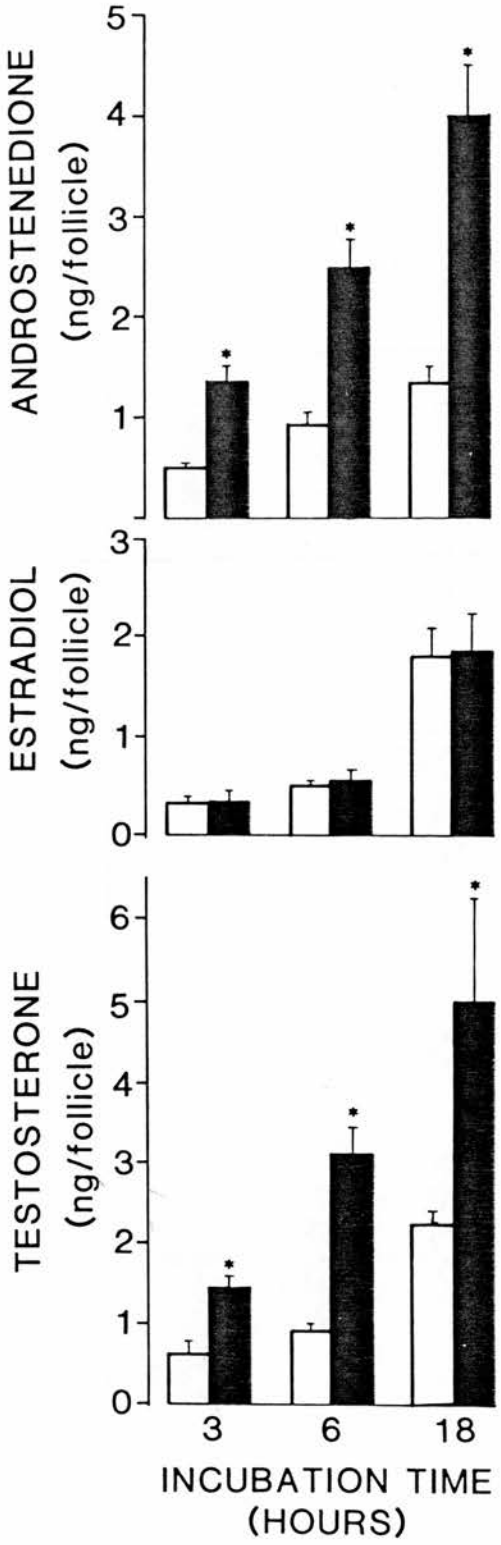

FIGURE 7.1 Androstenedione, testosterone and oestradiol release from isolated rat follicles incubated with (■) or without (□) 10^{-8}M LHRH agonist for periods of 3, 6 or 18 h.

Data shown are Mean ± S.E.M. (10 follicles per group).

* $P < 0.001$ for treated compared to control values at each time period.

unaffected by LHRH treatment at any time.

7.3.2 Release versus production of steroids

Method:- In order to investigate whether accumulation of steroids into the medium was the result of leakage of preformed steroids or due to net production i.e. de novo synthesis and release, 20 follicles were incubated, 10 with and 10 without 10^{-8}M LHRH agonist, for 6 h. After incubation the follicles were removed and their steroid content assessed by ethanol extraction. To assess tissue content prior to incubation 10 unincubated follicles were also subjected to ethanol extraction. After individual homogenization of follicles in 1 ml ethanol in a glass 1 ml homogenizer, the homogenate was left overnight at 4°C prior to drying the supernatant under nitrogen and reconstituting the residue in PGBS for the estimation of total tissue steroid content.

Results:- Follicular content of testosterone and oestradiol was low and unchanged by incubation in control medium. Tissue progesterone content was non-detectable ($\angle$ 50 pg/follicle) prior to incubation and just detectable (70 pg/follicle) after control incubation. Incubation with 10^{-8}M LHRH agonist resulted in a significant increase in both tissue content and released steroid for testosterone ($P \angle 0.001$) and progesterone ($P \angle 0.01$) but had no effect on oestradiol (Fig. 7.2). Since LHRH agonist treatment resulted in steroid levels greater than those found in pre-incubated tissue ($P \angle 0.001$ for testosterone, oestradiol and progesterone) it is clear that net production of steroids occurred during the incubation.

It is interesting that in control incubations the released steroid concentration was also in excess of that which could be explained on the

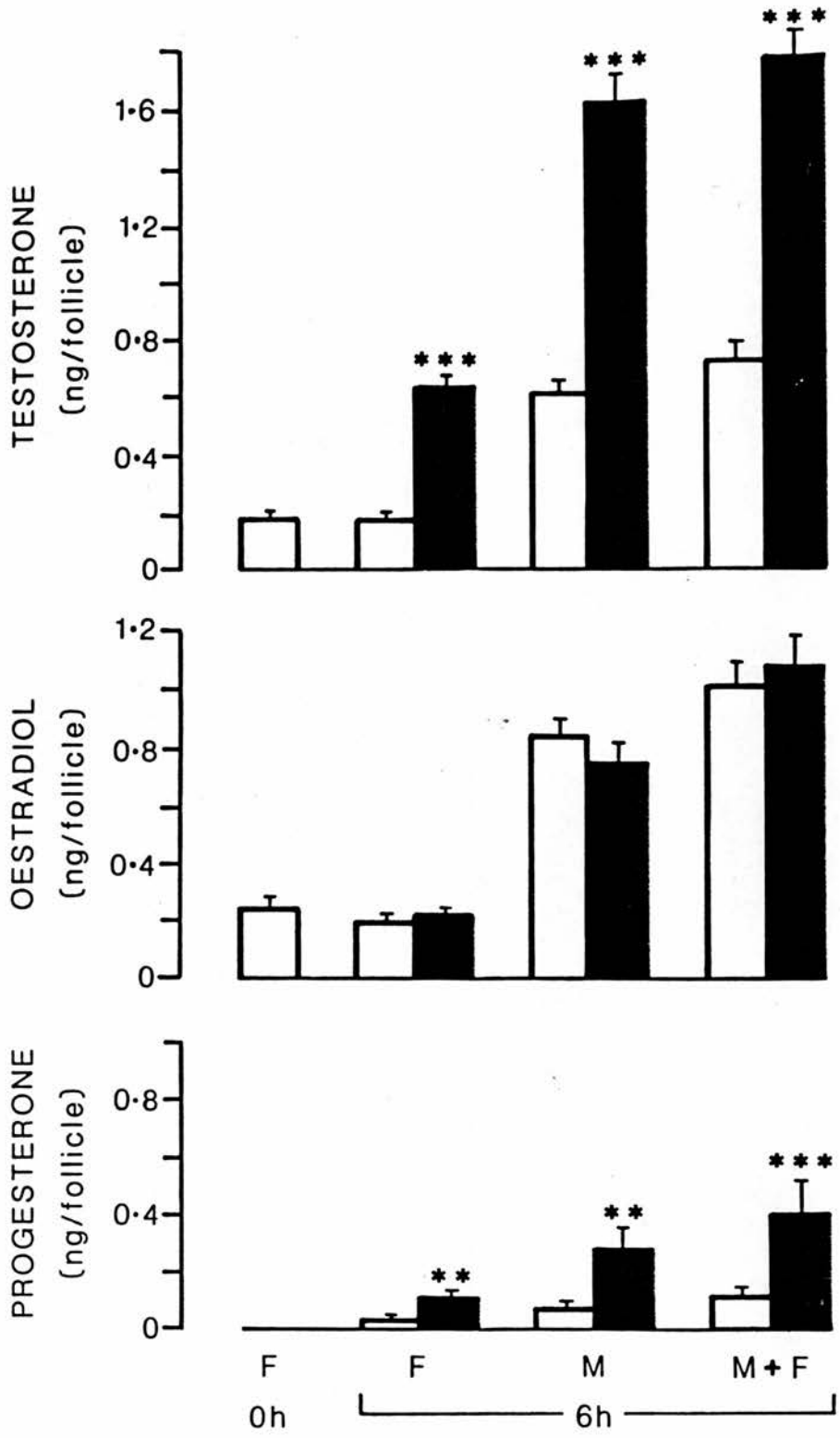

FIGURE 7.2 Testosterone, oestradiol and progesterone levels after incubation for 0 h or 6 h with (■) or without (□) 10^{-8}M LHRH agonist in ethanol extracted follicles (F), medium alone (M) or after freezing and thawing of both medium and follicles (M + F).

Data shown are Mean ± S.E.M. (10 follicles per group).

** $P < 0.01$; *** $P < 0.001$ for treated compared to control values in each pair of columns.

basis of passive release of preformed steroid indicating that de novo steroid synthesis had occurred in control follicles.

7.3.3 Freezing and thawing as a measure of tissue steroid content

Method:- Since freezing and thawing of follicles has been shown to result in the release of 98% of the tissue content of oestrogens (Carson et al., 1981) it was decided to compare the steroid content of medium after freezing and thawing of medium plus follicle, with that estimated from separate analyses of follicle and medium content. Twenty follicles were incubated for 6 h in the presence or absence of 10^{-8}M LHRH agonist (10 follicles per group) and the incubation terminated by freezing the incubation tray (medium + follicle) at -20°C prior to assay.

Results:- Freezing and thawing of medium plus follicle resulted in the liberation of the majority of tissue steroid content (Fig. 7.2; see Carson et al., 1981). No significant differences were found between the expected value for medium plus tissue content calculated after separate analysis, and that obtained after assay subsequent to freezing and thawing (values for estimated recovery of tissue content ranged from 78-100%). The results obtained after freezing and thawing thus gave essentially the same results as those estimated from separate analyses of medium and steroid content, namely net stimulation of androgen and progesterone production by LHRH agonist. Since oestradiol was unchanged, it was unlikely that the variability in oestradiol seen in subsequent experiments was due to insufficient release of tissue content. For convenience all subsequent experiments were therefore terminated by freezing the follicle and medium. The medium content of steroids was then considered to represent total released hormone plus the majority of tissue content.

7.3.4 Dose-dependent stimulation

7.3.4.1 LHRH agonist

Method:- In order to assess the dose-dependence of LHRH agonist stimulation 8 groups of 10 follicles were incubated with medium alone or with various doses of LHRH agonist for 18 h.

Results:- LHRH agonist stimulated production of androstenedione, testosterone and progesterone in a dose-dependent manner. The minimal stimulatory dose ($P < 0.001$) was 5×10^{-10}M (Fig. 7.3). Oestradiol production was variable and, although no dose dependent changes were seen, levels increased significantly over control values in the presence of 5×10^{-10}M LHRH agonist. Concentrations ranged from 6 $\pm$ 1 ng/follicle (control) to 7.5 $\pm$ 0.3 - 10.5 $\pm$ 1.5 ng/follicle (treated), mean $\pm$ SEM.

7.3.4.2 LHRH

Method:- Six groups of 10 follicles were incubated with medium alone or in the presence of various doses of LHRH for 18 h.

Results:- LHRH stimulated androgen and progesterone production in a dose-dependent manner with doses greater than 10^{-9}M (Fig. 7.4). Oestradiol levels ranged from 1.5 $\pm$ 0.9 ng/follicle (control) to 2.1 $\pm$ 1.2 ng/follicle (treated), mean $\pm$ SEM.

7.3.5 Time course of stimulatory action

Method:- Three groups of 10 follicles were incubated for 20 h with 10^{-8}M LHRH agonist, 10^{-6}M LHRH or medium alone for 20 h. Medium was replaced , containing the same hormonal additions after 2, 4, 6, 8 and 20 hrs.

Results:- The results were either expressed as cumulative steroid release (Fig. 7.5) or as steroid released during each incubation period

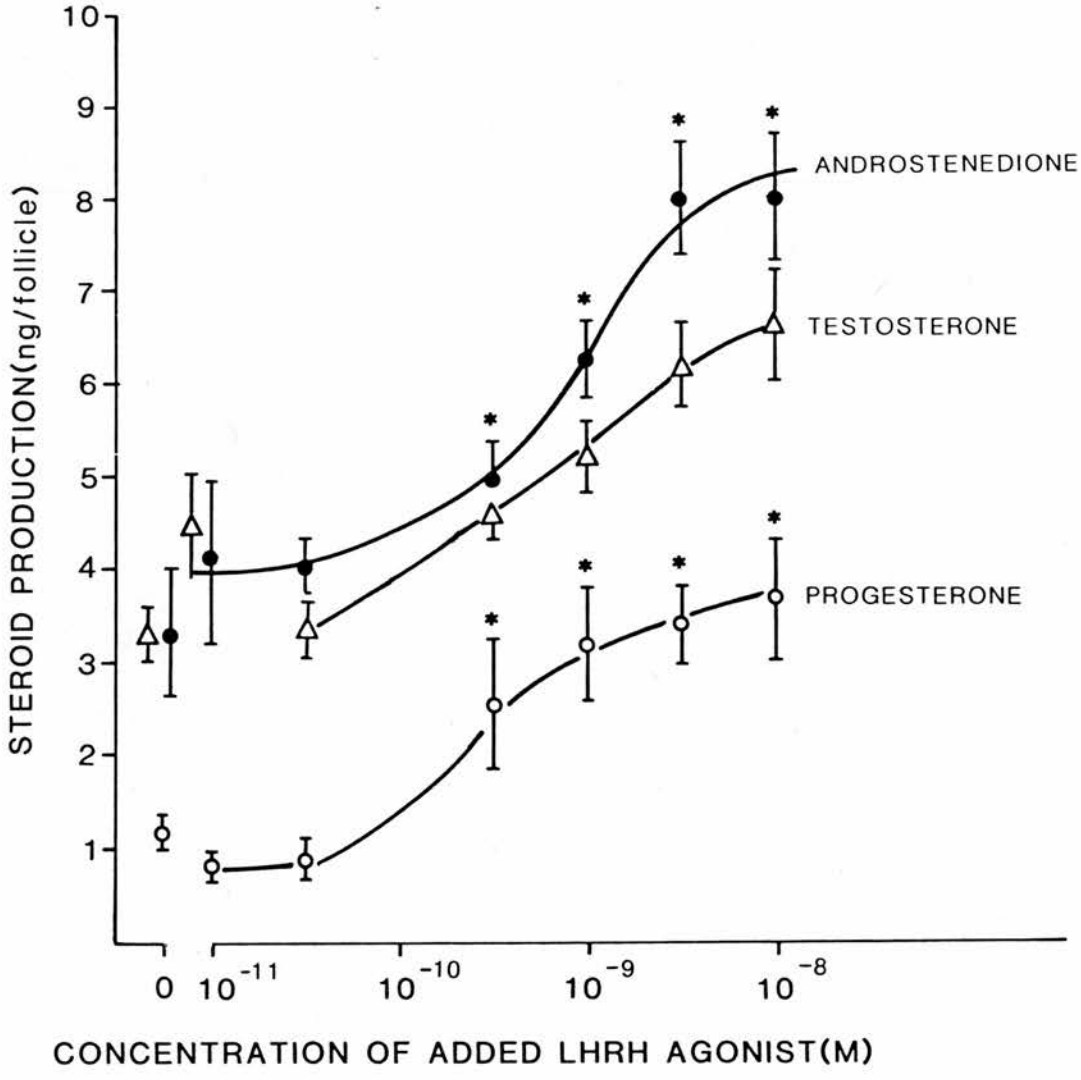

FIGURE 7.3 Dose-dependent stimulation of androstenedione (●—●), testosterone (△—△) and progesterone (○—○) production from follicles incubated with various concentrations of LHRH for 18 h.

Data are Mean ± S.E.M. (10 follicles per group).

* $P < 0.001$ compared to control value in absence of LHRH.

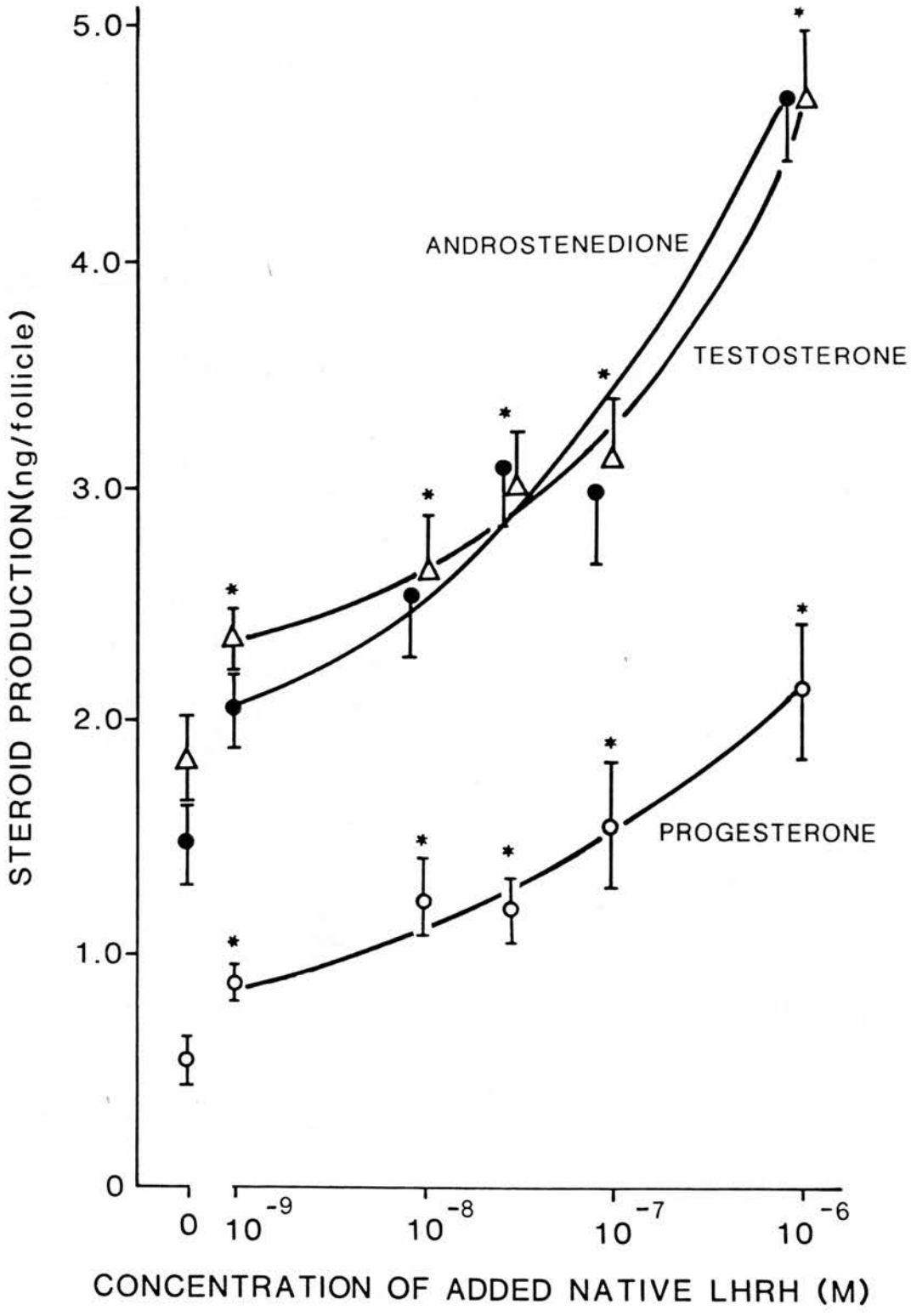

FIGURE 7.4 Dose dependent stimulation of androstenedione (●—●), testosterone (△—△) and progesterone (○—○) production from follicles incubated with various concentrations of LHRH for 18 h.

Data are Mean ± S.E.M. (10 follicles per group).

* $P < 0.001$ compared to control value in absence of LHRH.

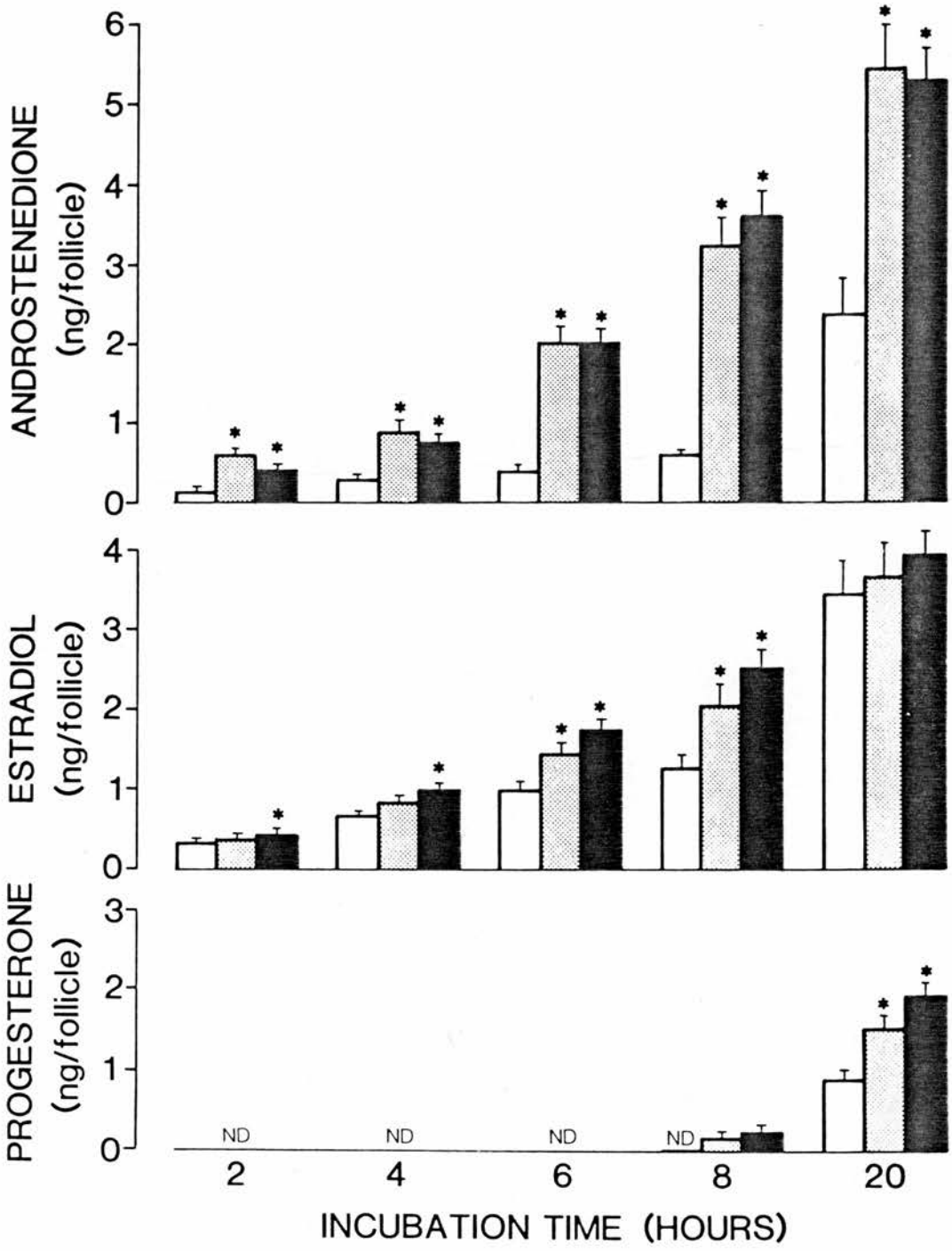

FIGURE 7.5 Time course of androstenedione, progesterone and oestradiol release from follicles incubated with 10^{-8}M LHRH agonist (■), 10^{-6}M LHRH (▨) or medium alone (□). Medium was replaced after 2, 4, 6, 8 and 20 h.

Data shown represent cumulative totals (Mean ± S.E.M., 10 follicles per group) of steroid production over the given time period.

ND = Non-Detectable.

* $P < 0.001$ for treated compared to control values at each time period.

TABLE 7.1

Androstenedione, testosterone, oestradiol and progesterone release from follicles incubated with 10^{-8}M LHRH agonist, 10^{-6}M LHRH or medium alone for 2 h periods up to 8 h and from 8-20 h.

Data are mean ± S.E.M. (10 follicles/group). Values differ from those of M199 Controls [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$; ND = Non-Detectable.

Time (h)	Steroid pg/follicle	Treatment		
		Control (M199)	10^{-6}M LHRH	10^{-8}M LHRH agonist
0-2	Androstenedione	140± 11	558± 60[a]	419± 58[a]
	Testosterone	365± 50	1079±101[a]	871± 51[a]
	Oestradiol	344± 22	361± 26	436± 21[b]
	Progesterone	ND	ND	ND
2-4	Androstenedione	167± 16	850±123[a]	844± 74[a]
	Testosterone	350± 20	1080± 91[a]	1074± 81[a]
	Oestradiol	338± 24	505± 53[b]	584± 58[a]
	Progesterone	ND	ND	ND
4-6	Androstenedione	133± 10	743± 79[a]	590± 54[a]
	Testosterone	360± 40	1409±109[a]	1052± 50[a]
	Oestradiol	256± 31	465± 81[c]	536± 60[a]
	Progesterone	ND	ND	ND
6-8	Androstenedione	188± 32	1226±154[a]	1553±176[a]
	Testosterone	329± 23	2179±190[a]	3021±220[a]
	Oestradiol	321± 45	605±113[c]	753±103[b]
	Progesterone	ND	198± 50	1260± 34
8-20	Androstenedione	1751±366	2167±255	1667±237
	Testosterone	4547±564	5485±622	3910±592
	Oestradiol	2131±363	1576±296	1415±131
	Progesterone	904± 90	1355±129[b]	1204±123[b]

(Table 7.1).

Androstenedione (Fig. 7.5; Table 7.1) and testosterone (Table 7.1) release were stimulated by 10^{-8}M LHRH agonist and 10^{-6}M LHRH during each 2 h period up to 8 h, resulting in significant accumulation after 2, 4, 6 and 8 h. The release of androgen from 8-20 h was not significantly greater than that of control over this time period (Table 7.1) but the cumulative total at 20 h was still increased (Fig. 7.5).

Progesterone was not detectable until after 6-8 h incubation when both LHRH and LHRH agonist raised concentrations to detectable levels. Both peptides stimulated progesterone release between 8-20 h (Table 7.1; Fig. 7.5).

Oestradiol release was significantly stimulated by 10^{-8}M LHRH agonist during each 2 h period up to 8 h, and by 10^{-6}M LHRH at 2-4 h, 4-6 and 6-8 h (Table 1) but not 8-20 h. The cumulative total showed no net effect of either peptide on oestradiol levels after 20 h.

A 100 fold higher concentration of LHRH (10^{-6}M) was required to cause a response similar to that achieved with 10^{-8}M LHRH agonist.

7.3.6 Comparison of LHRH-stimulated and hCG-stimulated steroidogenesis

Method:- In order to compare the stimulatory effects of LHRH with those of hCG, an experiment was performed similar to that described in 7.3.4, with 4 groups of 10 follicles being incubated with either medium alone, 10^{-8}M LHRH, 10^{-8}M LHRH agonist or 50 mIU hCG, for 20 h. Medium was replaced with fresh, containing the same hormonal additions, after 2, 4, 6, 8 and 20 h.

Results:- Incubation with 10^{-8}M LHRH agonist resulted in the same stimulatory effects seen previously, with both androstenedione and

TABLE 7.2

Androstenedione, oestradiol and progesterone release from follicles incubated with 10^{-8}M LHRH agonist, 10^{-6}M LHRH, 50 mIU hCG or medium alone for 2 h periods up to 8 h and from 8-20 h.

Data are mean + S.E.M. (10 follicles/group). Values differ from those of M199 Controls [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$; ND = Non Detectable.

Time (h)	Steroid pg/follicle	Treatment			
		Control (M199)	10^{-8}M LHRH	10^{-8}M LHRH Agonist	50 mIU hCG
0-2	Androstenedione	87± 7	78± 5	136± 29[a]	995± 74[a]
	Oestradiol	227± 18	266± 22	268± 13	472± 44[a]
	Progesterone	ND	ND	ND	189± 24
2-4	Androstenedione	118± 14	224± 32[b]	634± 83[a]	2759±391[a]
	Oestradiol	247± 17	294± 22	472± 48[a]	736±193[a]
	Progesterone	ND	ND	ND	400± 90
4-6	Androstenedione	144± 19	220± 26[c]	494± 71[a]	4169±293[a]
	Oestradiol	346± 27	330± 34	518± 61[b]	2014±299[a]
	Progesterone	ND	ND	ND	309± 67
6-8	Androstenedione	212± 24	234± 33	671±138[a]	2634±182[a]
	Oestradiol	279± 27	286± 25	570± 75[a]	1581±199[a]
	Progesterone	ND	ND	ND	237± 48
8-20	Androstenedione	1059±220	677± 41[b]	1312±260	1089±142
	Oestradiol	1440±378	620± 90	1510±284	1307±239
	Progesterone	400± 50	685± 58[b]	858± 73[a]	960±110[a]

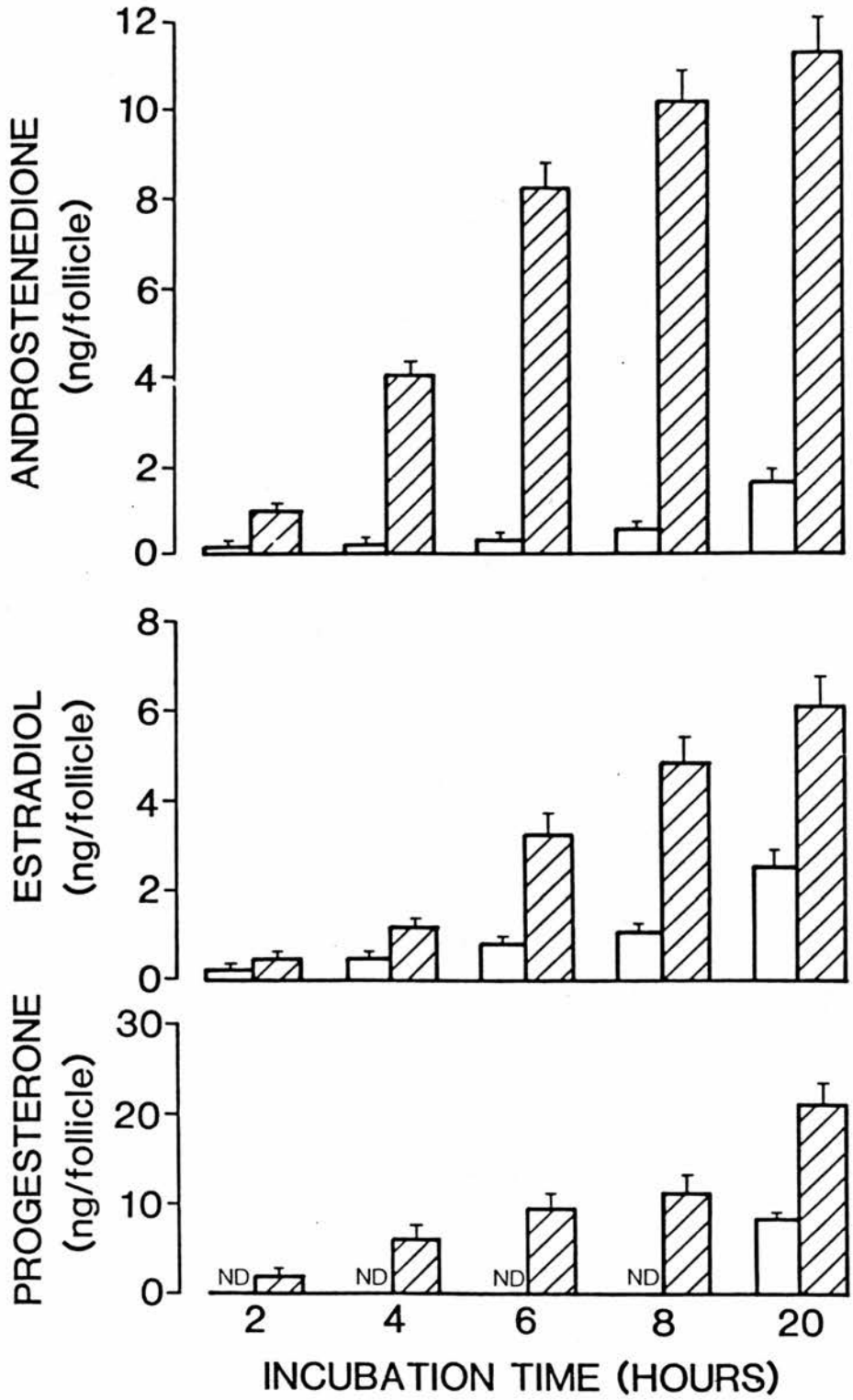

FIGURE 7.6 Time course of androstenedione, progesterone and oestradiol release from follicles incubated either with (▨) or without (□) 50mIU hCG. Medium was replaced at 2, 4, 6, 8 and 20 h.

Data represents cumulative totals (Mean ± S.E.M., 10 follicles per group) of steroid production over each time period.

ND = Non Detectable.

oestradiol release being stimulated at each 2 h time period upto 8 h. A lower dose (10^{-8}M) of LHRH was used, so as expected, the stimulatory effect on androstenedione was less than that of 10^{-8}M LHRH agonist, and oestradiol release was unaffected (Table 7.2).

50 mIU hCG resulted in significant stimulation of all steroids (androgen, oestradiol and progesterone) at each 2 h time point up to 8 h (Table 7.2; Fig. 7.6). This is in contrast to the stimulation with LHRH where progesterone release was only stimulated after 6-8 h incubation (Tables 7.1; 7.2). However, in agreement with the actions of LHRH, androgen and oestradiol release were not observed during 8-20 h incubation with hCG, whereas net progesterone release continued to occur (Tables 7.1; 7.2).

7.3.7. Summary

The experiments presented so far have shown:-

(1) LHRH and LHRH agonist stimulated basal androgen and progesterone production.

(2) LHRH agonist was approximately 100 times more potent than LHRH.

(3) Stimulation was dose-dependent, the minimal effective doses being 5 x 10^{-10}M for LHRH agonist, and 10^{-9}M for LHRH.

(4) Stimulation of androgen production by LHRH agonist was immediate (2-3 hrs), whereas that of progesterone was delayed (6-8 hrs).

(5) The magnitude of stimulation was small in comparison to that of 50 mIU hCG.

(6) Oestradiol production varied between experiments; either a stimulation of production occurred or no change occurred from basal levels.

7.4 CHARACTERISTICS OF LHRH AGONIST STIMULATION

7.4.1 Effect of pre-incubation

Studies using rat Leydig cells had indicated that hCG-induced stimulation of testosterone production would occur over a 4 h incubation period in hormone-free medium subsequent to a 15 minute exposure to hCG (Sharpe & Cooper, 1982a). Thus the continued presence of gonadotrophin was not essential for its stimulatory effects on steroidogenesis. The following experiment set out to investigate whether this was the case for LHRH agonist-stimulated steroid production in isolated rat follicles.

Method:- One Group of 20 follicles was incubated in the presence or absence of 10^{-8}M LHRH agonist, for 18 h. Another group of 20 follicles was pre-incubated for 3 h with LHRH agonist or medium alone before being transferred to hormone-free medium for 18 h incubation.

Results:- The results shown in Fig. 7.7 indicate that after 18 h continuous incubation with 10^{-8}M LHRH agonist, androstenedione and progesterone release were stimulated ($P < 0.001$). Incubation for 3 h resulted in increased androstenedione release in the LHRH agonist-treated follicles. Despite pre-incubation for 3 h with LHRH agonist, steroid production during the subsequent 18 h period in medium alone was not significantly different from the control value.

The continued presence of LHRH agonist was therefore necessary for stimulation of basal steroid production.

7.4.2 Specificity of action

The ability of non-LHRH-related peptides to stimulate basal steroidogenesis was investigated.

Method:- Four groups of 10 follicles were incubated for 3 h followed

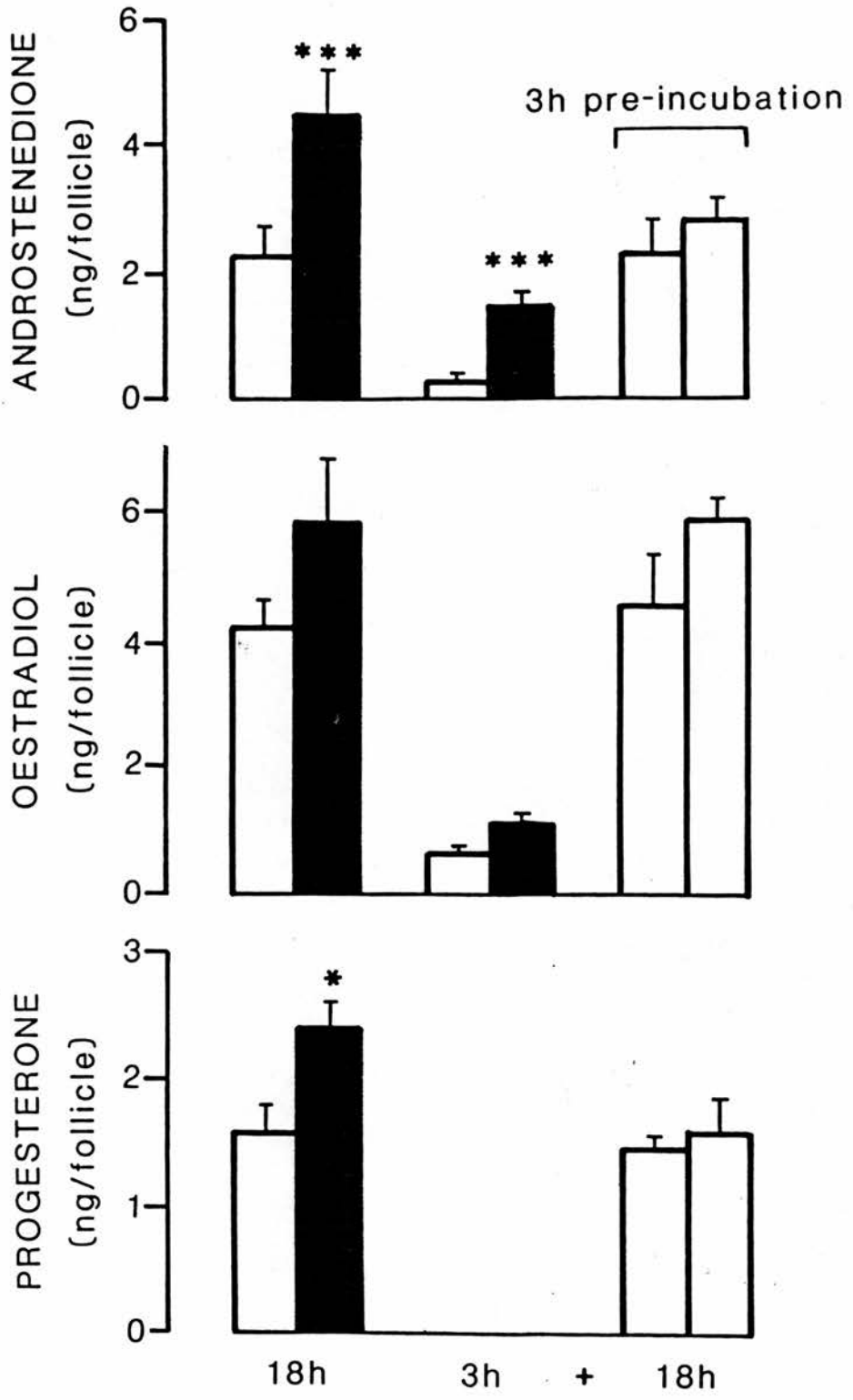

FIGURE 7.7 Androstenedione, testosterone and oestradiol production from follicles incubated in the presence (■) or absence (□) of 10^{-8}M LHRH agonist for 18 h or 3 h. After 3 h pre-incubation, follicles were transferred to medium alone for 18 h (Right hand columns).

Data are Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; *** $P < 0.001$ for treated compared to control values at each time period.

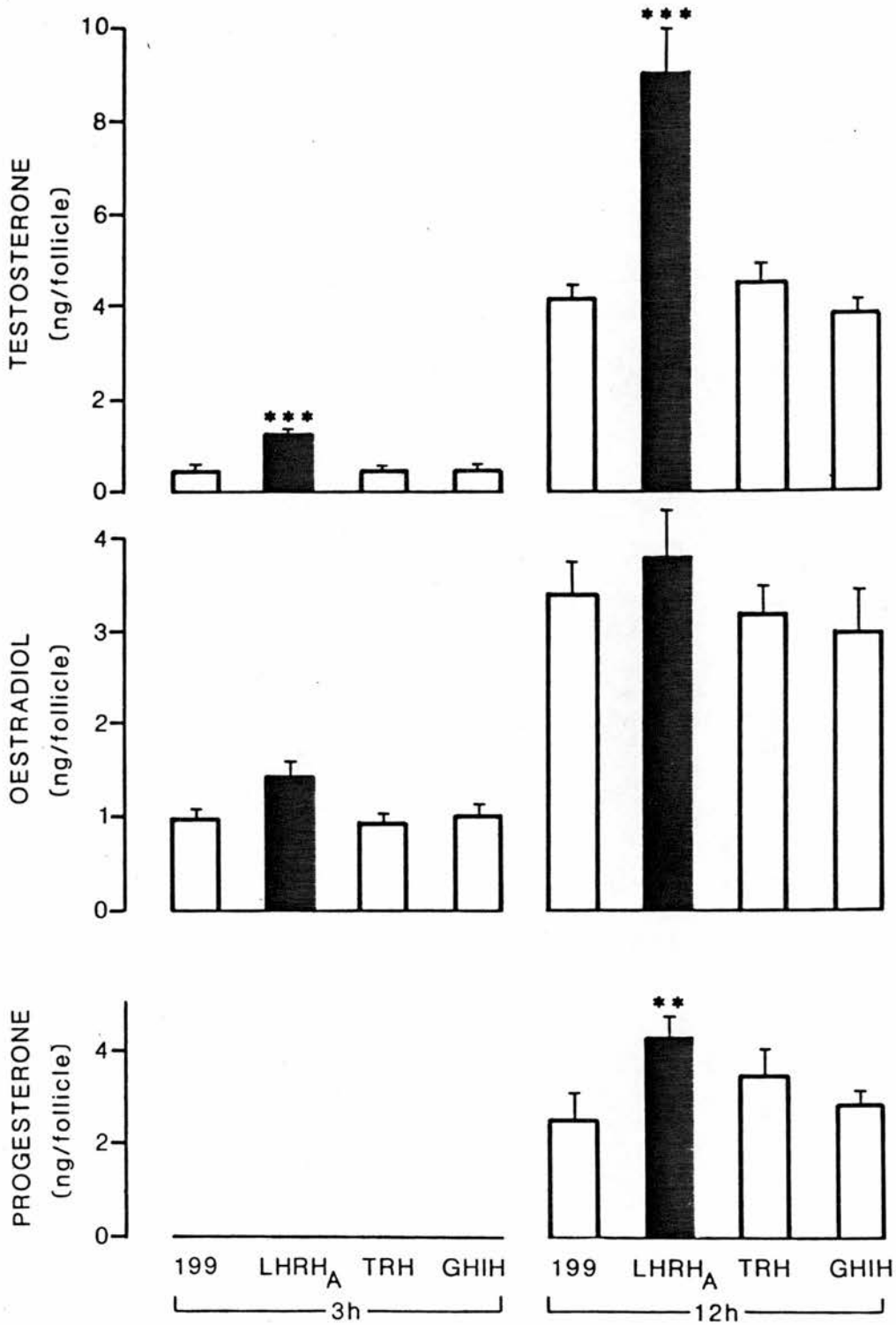

FIGURE 7.8 Testosterone, progesterone and oestradiol production from follicles incubated for 3 h followed by 17 h in medium alone (199) or in the presence of 10^{-8}M LHRH agonist (LHRH$_A$), 1000ng thyrotrophin releasing hormone (TRH) or 1000 ng somatostatin (GHIH).

Data are Mean $\pm$ S.E.M. (10 follicles per group).

** $P < 0.01$; *** $P < 0.001$ for treated compared to control values at each time period.

by 12 h with medium alone, 10^{-8}M LHRH agonist, 1000 ng TRH or 1000 ng somatostatin (GHIH).

Results:- 10^{-8}M LHRH agonist significantly stimulated basal testosterone release after 3 h ($P < 0.001$), and basal testosterone ($P < 0.001$) and progesterone ($P < 0.01$) production after 12 h. Neither TRH or GHIH influenced basal steroid production (Fig. 7.8).

This result agrees with the LHRH receptor data (see Chapter 3) indicating specificity of both the receptors and the steroidogenic response for LHRH agonist. Non-related peptides did not interact with LHRH receptors, nor did they influence basal steroidogenesis.

7.4.3 Effect of an LHRH antagonist

In order to assess further the specificity of LHRH actions, the effect of an LHRH antagonist (DPhe^2 Phe^3 DPhe^6 LHRH) on LHRH- and LHRH agonist-induced changes was examined.

Method:- Three groups of 20 follicles were incubated with medium alone, 10^{-8}M LHRH agonist or 10^{-6}M LHRH, in the presence or absence of 2 µg (4×10^{-6}M) LHRH antagonist for 3 h followed by 17 h.

Results:- Basal steroid production was unaffected by LHRH antagonist, so the results from each group were pooled to give one control group. The results for testosterone and progesterone production are expressed as percentage increment over control values (Fig. 7.9). LHRH and LHRH agonist induced the expected stimulation of testosterone release after 3 h and 17 h, and of progesterone after 17 h incubation. Concomitant treatment with LHRH antagonist reduced the stimulatory effects of both molecules, being most potent in reducing the testosterone reponse to 10^{-6}M LHRH after 3 h ($P < 0.001$) (Fig. 7.9). Since only a partial

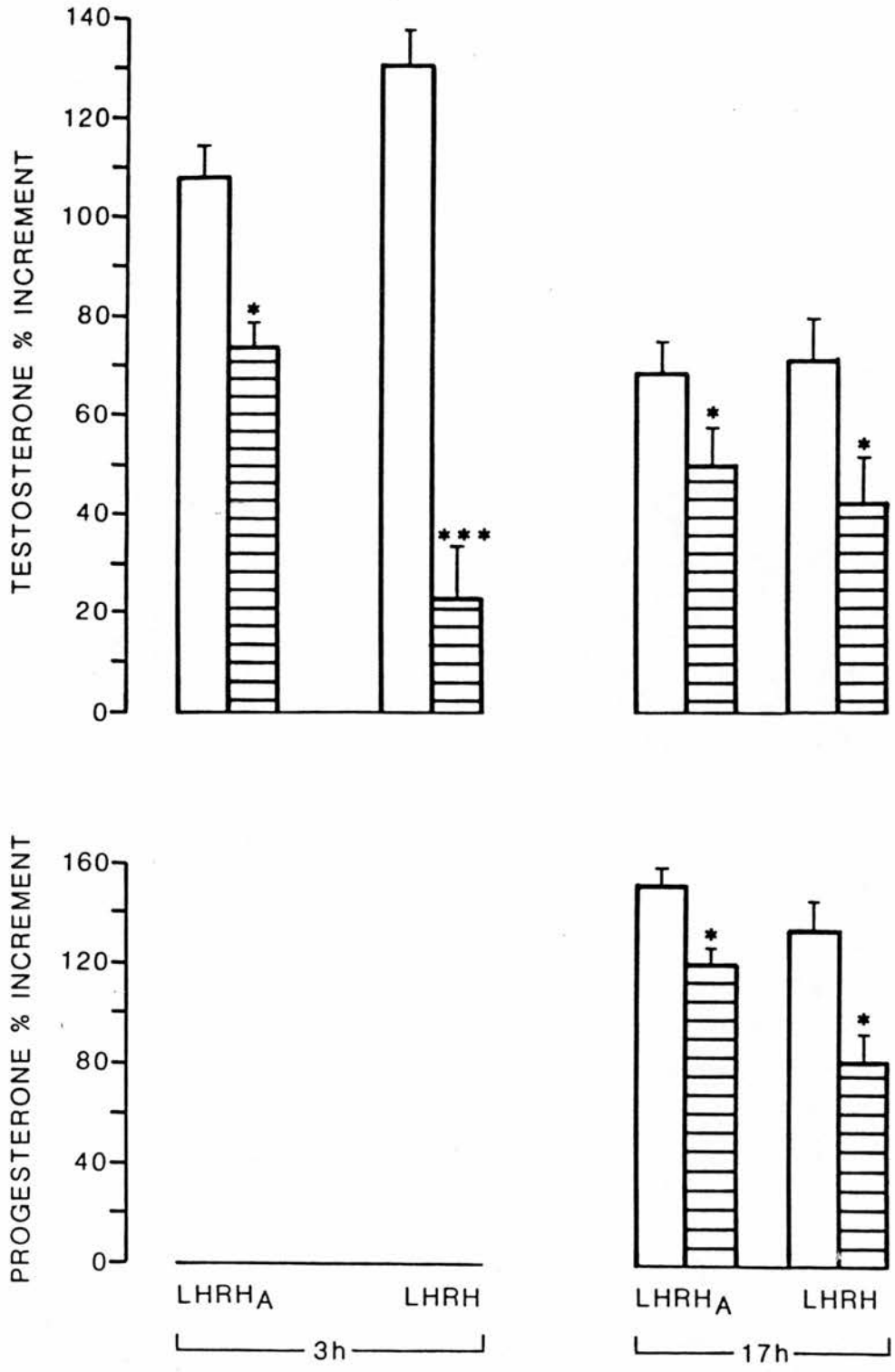

FIGURE 7.9 Percentage increment in testosterone and progesterone production from follicles after 3 h followed by 17 h incubation with 10^{-8}M LHRH agonist (LHRH$_A$) or 10^{-6}M LHRH in the presence (▤) or absence (□) of 2 μg LHRH antagonist.

Data are Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; *** $P < 0.001$ for antagonist treated compared to Non-antagonist treated values at each time period.

inhibition was observed it could be suggested that the antagonist analogue used was not potent enough to completely prevent interaction of LHRH with its receptors.

Results from this and the previous experiment emphasise the specific nature of the direct gonadal effects of LHRH and confirm receptor data presented in Chapter 3.

7.4.4 Summary

(1) The continued presence of LHRH agonist was essential for expression of its stimulatory effects.

(2) Stimulation was specific for LHRH and LHRH agonist; unrelated peptides such as TRH and GHIH had no effect.

(3) LHRH antagonist reduced the stimulatory effects of LHRH and LHRH agonist.

7.5 DOES LHRH AGONIST INHIBIT AROMATASE ?

Since oestradiol results were variable between experiments it was pertinent to investigate the possibility that the stimulatory effects of LHRH on basal androgen production were due to an inhibitory effect on the granulosa cell enzyme aromatase (which converts testosterone to oestradiol), thus resulting in a passive build up of substrate by the thecal cell. Since aromatase activity is located exclusively in the granulosa cells of the rat (Dorrington, 1975; Erickson & Hsueh, 1978) and depends entirely on thecal androgen as its sole precursor, the ability of LHRH agonist to influence the conversion of exogenously added testosterone to oestradiol, was assessed in isolated granulosa cells.

7.5.1 Studies with isolated granulosa cells

Method:- Granulosa cells were isolated from ovaries of immature

rats killed 48 h after the administration of 4 IU PMSG, by puncturing the follicles with a fine needle to expel the cells. After centrifugation at 700 rpm for 15 minutes at 37°C and washing (with 3 x 1 ml incubation medium) the cells were counted (viability, as assessed by trypan blue exclusion, was 70-80%) and incubated in multiwell plates with 2.5×10^{-5} cells per well, as described for follicles, for 4 h. Several inhibitors of aromatase have been used previously but 1 µg 5 α dihydrotestosterone (DHT) has been suggested as the most effective (Hillier et al., 1980). The effect of 10^{-8}M LHRH agonist on the conversion of a range of doses of testosterone to oestradiol was assessed and compared with that of DHT. Six groups of 10 wells (with 2.5×10^{-5} cells/well) were incubated with 0, 5, 10, 25, 50 or 100 ng testosterone in the presence or absence of 10^{-8}M LHRH agonist. An additional group of 10 wells was incubated with 100 ng testosterone and 1 µg 5 α DHT in the presence and absence of 10^{-8}M LHRH agonist.

Results:- Testosterone resulted in a dose-dependent increase in oestradiol production (Fig. 7.10). The oestradiol response was not significantly affected by 10^{-8}M LHRH agonist except in the presence of 100 ng testosterone, when LHRH agonist stimulated apparent aromatase activity ($P < 0.05$). The potent aromatase inhibitor, 5 α DHT, inhibited ($P < 0.05$) the conversion of 100 ng testosterone to oestradiol in the presence and absence of LHRH agonist.

LHRH agonist clearly had no inhibitory effect on the conversion of testosterone to oestradiol, and did not behave in a manner similar to that of an aromatase inhibitor.

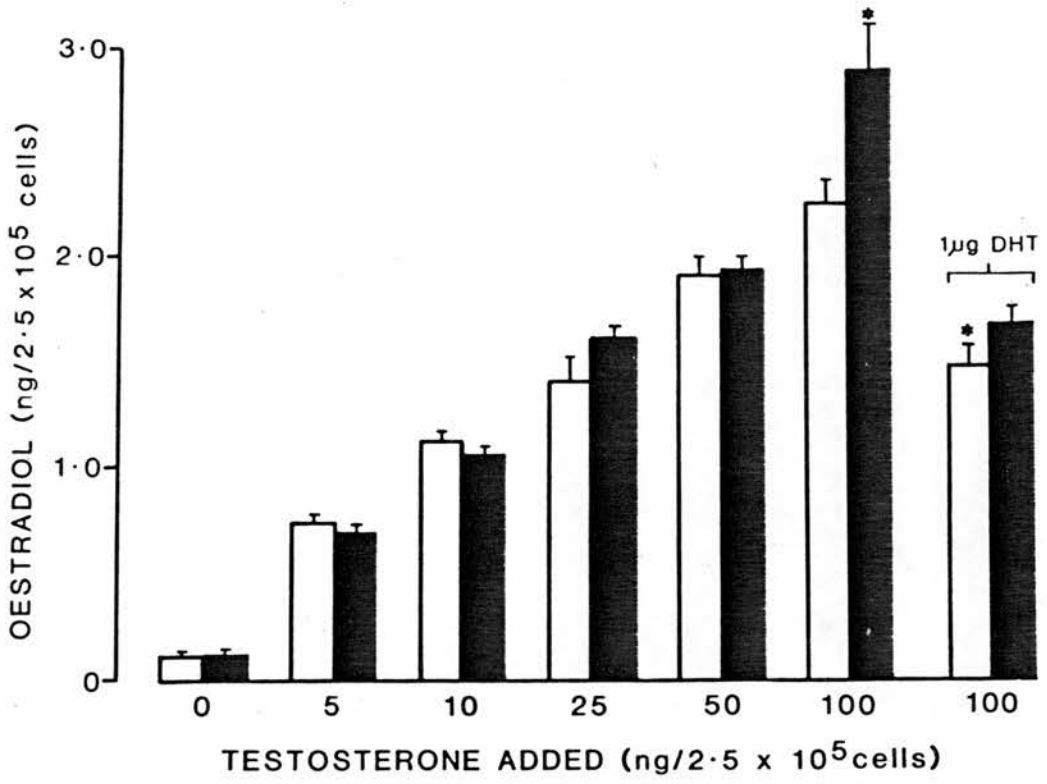

FIGURE 7.10 Oestradiol production from isolated granulosa cells incubated for 4 h with various doses of testosterone and in the presence of an aromatase inhibitor (1 μg DHT) with (■) and without (□) 10^{-8}M LHRH agonist.

Data are Mean ± S.E.M. (5 observations per group).

* $P < 0.05$ for treated compared to control values at each dose of testosterone added.

TABLE 7.3

Oestradiol production (pg/follicle) from isolated pre-ovulatory follicles incubated for 3 h and 17 h with M199, 10^{-8}M LHRH agonist, 100 ng testosterone, 1 μg 5∝ dihydrotestosterone (DHT) as shown.

Data represents mean ± S.E.M. with 10 follicles per group. Values differ from M199 control or as indicated. [a] $P < 0.001$; [b] $P < 0.01$

	Time (h) 3 h	17 h
Control (M199)	333±33	821± 90
100 ng Testosterone	523±45[b]	1910±275[a]
100 ng Testosterone + 1 μg 5∝ DHT	487±60	928±120 [b]
1 μg 5∝ DHT	307±37	745± 99
10^{-8}M LHRH Agonist	420±70	1660±130[a]
10^{-8}M LHRH Agonist + 5∝ DHT	457±39	1164±115 [b]
10^{-8}M LHRH Agonist + 100 ng Testosterone	643±66[a]	2081±215[a]

7.5.2 Studies with whole follicles

Attempts at examining the effects of LHRH agonist on androgen production in the presence of the aromatase inhibitor 5 ∝ DHT proved unsuccessful. The rationale behind the experiments was to ascertain whether inhibition of basal aromatase could be achieved in isolated follicles by 5 ∝ DHT, and whether this would result in an increase in androgen similar to that seen with LHRH. In addition it was hoped to utilize this model to investigate whether LHRH would induce a further increase in androgen production in the presence of 5 ∝ DHT, thus indicating a direct thecal site of action for LHRH.

Method:- Seventy follicles were incubated (10 follicles per group) for 3 h and 17 h with medium alone, 10^{-8}M LHRH agonist, 100 ng testosterone (T), 1 μg 5 ∝ dihydrotestosterone (DHT), LHRH agonist plus 100 ng T, 100 ng T plus 1 μg 5 ∝ DHT or LHRH agonist plus 1 μg DHT.

Results:- Oestradiol production is shown in Table 7.3. Oestradiol production was increased in the presence of 100 ng T at 3 h and 17 h ($P < 0.01$ and $P < 0.001$ respectively; Table 7.3) and this increase was inhibited ($P < 0.01$) by 1 μg 5 ∝ DHT after 17 h incubation. Basal oestradiol levels were unaffected by 5 ∝ DHT at 3 h or 17 h. 10^{-8}M LHRH agonist had no effect on the oestradiol produced in the presence of 100 ng T. Basal oestradiol levels were increased after 17 h incubation with LHRH agonist ($P < 0.001$) and this increase was inhibited by 5 ∝ DHT ($P < 0.01$).

These results confirm that the results seen in isolated granulosa cells were applicable to isolated follicles, namely that LHRH agonist failed to inhibit conversion of testosterone to oestradiol whereas a

potent aromatase inhibitor, 5 αDHT, reduced oestradiol production.

Since 1 μg 5 α DHT cross-reacted in the testosterone and androstenedione assays, even after attempts at removing the inhibitor by column chromatography, it was not possible to assess whether LHRH agonist stimulated thecal androgen production in the presence of the aromatase inhibitor.

7.5.3 Intact vs broken follicles

One other possible reason for the observed variability in the oestradiol response of isolated follicles to LHRH was that LHRH may be needed to exert direct granulosa cell effects and may be hindered by having to be transported through thecal cells to the inner granulosa cell layer. This could also be the reason why effects on granulosa progesterone production were seen only after 6-8 h whereas those on thecal androgen production were apparent after 2-3 h. The following experiment attempted to investigate this by comparing the steroidogenic response to LHRH agonist stimulation of intact and broken follicles.

Method:- Two groups of 20 follicles were incubated in the presence or absence of 10^{-8}M LHRH agonist. One group was incubated intact. In the second group, follicles were ruptured using a fine needle while in their individual wells. Incubations were carried out for 6 h.

Results:- Whole follicles showed the expected increased androgen ($P < 0.001$) and progesterone ($P < 0.05$) production in response to LHRH agonist but oestradiol levels were unaffected (Fig. 7.11). Broken follicles showed a similar androgen response ($P < 0.001$), although the response of granulosa cell parameters differed. Progesterone production

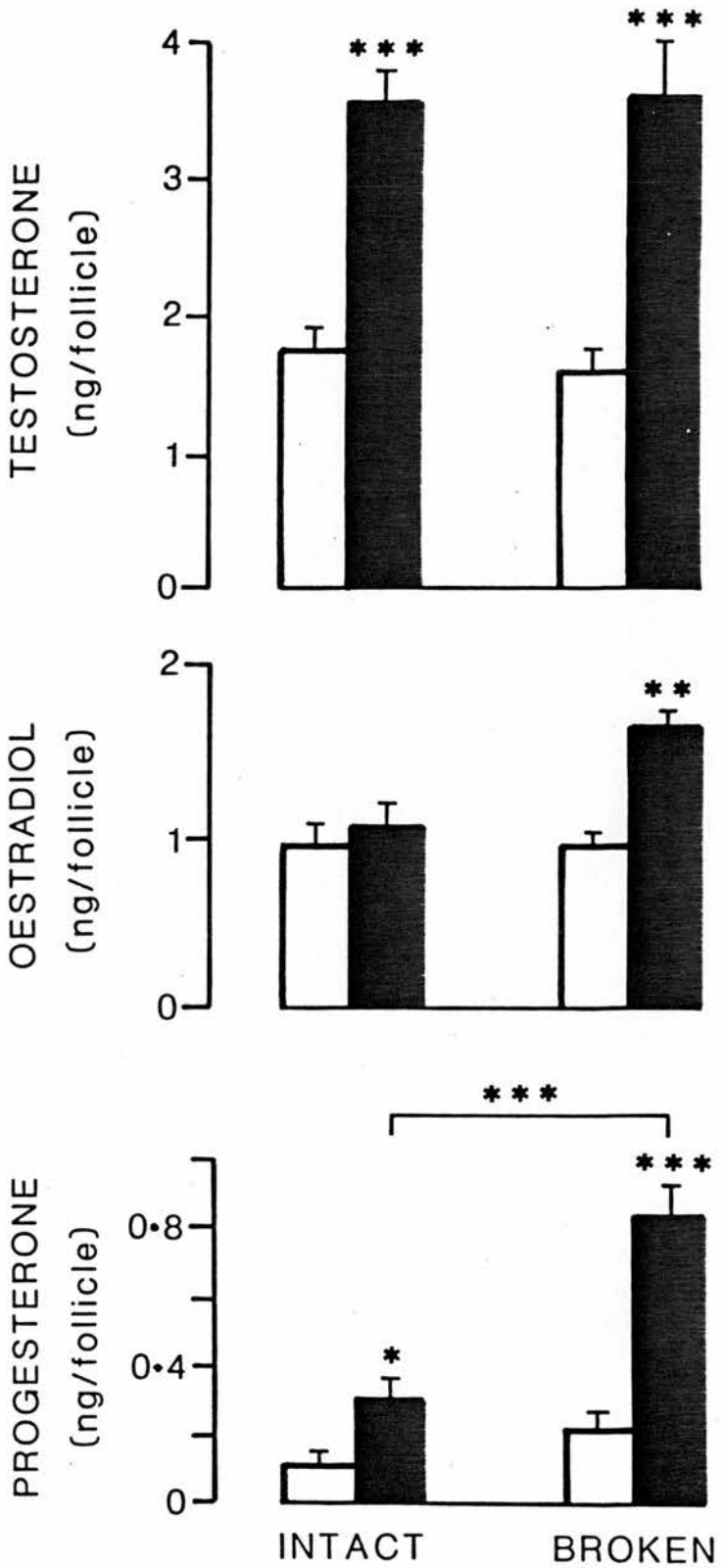

FIGURE 7.11 Testosterone, oestradiol and progesterone production from isolated intact or broken follicles incubated for 6 h in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data are Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; *** $P < 0.001$ for LHRH agonist treated compared to controls in each group, or as indicated.

in response to LHRH agonist was markedly enhanced compared with whole follicles ($P < 0.001$) and interestingly, oestradiol production was stimulated ($P < 0.002$). This is unlikely to be due to release of follicular fluid oestradiol since freezing and thawing liberated 90% oestradiol in both instances (see 7.3.2.2).

Thus LHRH agonist stimulated oestradiol production in broken but not intact follicles. It could be suggested therefore that in the broken follicles exogenous LHRH had immediate access to granulosa and thecal cells, enabling stimulation of granulosa cell oestradiol production in the presence of stimulated thecal cell androgen production.

7.5.4. Summary

(1) LHRH agonist had no effect on the conversion of testosterone to oestradiol whereas an aromatase inhibitor (5 α DHT) reduced oestradiol production.

(2) 5 α DHT failed to reduce basal oestradiol release from isolated follicles.

(3) Oestradiol release was stimulated by LHRH agonist after incubation with ruptured, but not intact follicles.

7.6 DISCUSSION

7.6.1 Characteristics of LHRH stimulation of basal steroidogenesis

10^{-8}M LHRH agonist consistently stimulated basal androstenedione and testosterone release from isolated follicles over incubation periods from 2-20 h, resulting in a 2-5 fold stimulation over basal levels. The response to LHRH was dose- and time-dependent, significant stimulation occurring with concentrations of LHRH agonist in the range 5×10^{-10}M to 10^{-8}M and with concentrations of native LHRH in the range

10^{-9} - 10^{-6}M. The greater potency of the agonist molecule was also indicated by the 100 fold higher concentration of native hormone (i.e. 10^{-6}M) required to elicit stimulation comparable to that found with 10^{-8}M agonist. A similar phenomenon has been observed by others (e.g. Hillier et al., 1981) and is likely to be due to increased resistance to degradation or to an increased binding affinity of the analogue. The concentrations required to elicit stimulation were in the same range as the Ka for ovarian LHRH receptors (e.g. Pieper et al., 1981 and Chapter 3) suggesting that the effects were receptor mediated.

Dose-dependent stimulation of progesterone release occurred with both LHRH and LHRH agonist after 18 h continuous exposure to the peptides. No detectable stimulation of progesterone release was seen until after 6-8 h of incubation. This is a longer period of incubation than that reported by others, who found increases in progesterone production after 4-6 h incubation with LHRH, both in isolated follicles (Hillensjö, 1981; Hillensjö et al., 1982) and granulosa cells (Clark, 1982). The reason for this difference is not known but may be related to the use of increased numbers of follicles or cells during incubation with LHRH.

Basal oestradiol production in response to LHRH varied between experiments. In some instances significant stimulatory effects were detected while in others no change was observed.

This pattern of stimulation of basal steroidogenesis was specific for LHRH since other peptides (e.g. TRH, GHIH) showed no effect. In addition the effect on testosterone and progesterone production could be inhibited using an LHRH antagonist.

The nature of the stimulatory effect of LHRH, compared with that of

hCG, is interesting. Both hormones stimulated testosterone and oestradiol production for periods up to 8 h, after which the follicles appeared to lose the ability to respond to either hCG or LHRH. However, progesterone release continued to occur after 8 h continuous exposure to either hormone. These changes could be due to the incubation conditions, with prolonged incubation resulting in receptor changes and subsequent switching of action from a predominantly thecal to a predominantly granulosa site, perhaps due to luteinization-like changes in granulosa cells.

However, LHRH action differed from that of hCG in two parameters. LHRH-stimulated progesterone production was evident only after an incubation period of 6-8 h whereas incubation with hCG resulted in immediate (2 h) stimulation of progesterone production. In addition, whereas hCG-stimulated steroid production continued after pre-incubation period (see Sharpe & Cooper, 1982a) removal of LHRH agonist from the incubation medium abolished the stimulatory effects of the peptide. Thus, the continued presence of LHRH is required to elicit the stimulatory effects on steroidogenesis. These differences between hCG- and LHRH-stimulated steroidogenesis may reflect the characteristics of the hormone-receptor interaction, that for LHRH being rapid ($t_{\frac{1}{2}}$ 20 minutes at 21° C) and that for hCG being slow ($t_{\frac{1}{2}}$ 150 minutes at 37° C) as suggested by Sharpe & Cooper, (1982a). It could be postulated that the delayed progesterone response to LHRH is due to a prolonged period required to activate functional granulosa cell LHRH receptors. It is evident, however that the characteristics of LHRH-stimulated steroidogenesis, differ from those of hCG.

7.6.2 Possible sites of action for LHRH stimulation

Stimulation of basal progesterone production has been reported from isolated granulosa cells (Clark et al., 1980). Since specific receptors for LHRH have been demonstrated to be present on isolated granulosa cells (e.g. Jones et al., 1980) the most likely explanation for LHRH-induced progesterone production in isolated follicles is that the increase is due to a direct action of LHRH on granulosa cells.

However, since androgens can stimulate progesterone production (Lucky et al., 1977; Nimrod, 1977) it is possible that LHRH-induced progesterone production from isolated follicles could be an indirect action via increased thecal androgen production. In addition, since thecal cells produce small amounts of progesterone (Terranova et al., 1982) a thecal source of progesterone cannot be excluded.

The mechanism by which LHRH-stimulated androgen production could occur is less clear. Since in the rat, thecal cells provide the major source of androgens (Fortune & Armstrong, 1977; Hamberger et al., 1978) it is possible that LHRH exerts a direct action on thecal cells. Indirect evidence suggested that specific receptors for LHRH may be present on thecal cells. Thus LHRH was found to bind to residual follicular tissue (presumably thecal/interstitial tissue) following the removal of granulosa cells (Pieper et al., 1981). In addition autoradiography revealed the presence of ^{125}I-LHRH agonist grains over thecal tissue (Chapter 3). Moreover LHRH agonist had been reported to influence interstitial cell steroidogenesis. (Magoffin et al., 1981).

Although there is evidence for granulosa cell production of androgens in some species e.g. hamster (Makris & Ryan, 1980), this is unlikely to be

the case in the rat where there is no evidence for granulosa cell androgen production.

Finally an apparent increase in androgen release could theoretically be caused by an inhibition of granulosa cell aromatization resulting in accumulation of precursor. While it is not possible to rule out this possibility due to the variability of the oestradiol response to LHRH, it seems unlikely for a number of reasons. Firstly, LHRH had no inhibitory effect on aromatase activity in isolated granulosa cells. Indeed recent studies have indicated that LHRH might stimulate aromatase activity (Dorrington et al., 1982). Secondly, in no experiment did LHRH show an inhibitory effect on basal aromatase; either no effect or a significant stimulation was observed. It could be suggested that in the model system used, under certain circumstances the aromatase enzyme may be incapable of responding to such relatively small changes in substrate concentration, especially since even known stimulators of aromatase (e.g. db cAMP) on occasion failed to stimulate oestradiol production (see 7.8.1).

One possibility investigated was that, in order for oestradiol production to occur in response to LHRH agonist-induced androgen production, granulosa cells required direct exposure to LHRH agonist. Since granulosa cells are separated from the outer medium by thecal cells, perhaps LHRH transport to the centre of the follicle was impaired. This could also explain the delayed progesterone increase. Oestradiol production was stimulated by incubation of LHRH agonist with broken but not intact follicles. Thus an additional effect of LHRH on the ability of granulosa cells to aromatize androgen cannot be excluded.

Attempts at stimulating androgen production with LHRH agonist in the

presence of an aromatase inhibitor proved unsuccessful due to interference of the inhibitor in androgen radioimmunoassays. Several groups have reported data utilizing isolated rat thecal preparations prepared from rat pre-ovulatory follicles (e.g. Fortune & Armstrong, 1977 & 1978; Hamberger et al., 1978). However, attempts at preparing enriched cultures of rat thecal cells proved unsuccessful due to technical difficulties and granulosa cell contamination.

Thus, although the data presented implies a direct thecal action of LHRH, until these observations are confirmed in isolated thecal preparations the question of direct thecal effects of LHRH remains a hypothesis only. Interestingly however, in a recent paper by Hillensjö et al., (1983) experiments were cited as demonstrating a direct stimulatory effect of LHRH on isolated rat thecal cells (Nordenstrom - in preparation, cited in Hillensjö et al., 1983).

7.7 EFFECTS OF LHRH AGONIST ON hCG-INDUCED STEROIDOGENESIS

7.7.1 Effect on hCG dose response

The stimulatory effects of LHRH and LHRH agonist on basal steroidogenesis were small in comparison to those seen with 50 mIU hCG (see 7.3.5). Since LHRH had been shown to decrease the sensitivity of granulosa cells to gonadotrophin stimulation in long-term culture (see Chapter 1 & Hsueh & Jones, 1981 for review) the ability of LHRH to influence hCG sensitivity in short-term culture was assessed.

Method:- Six groups of 20 follicles were incubated with either medium alone, 1, 5, 10, 25 or 50 mIU hCG in the presence or absence of 10^{-8}M LHRH agonist. After 3 h, medium was removed to assess steroid release and replaced with fresh medium with the same hormone additions and

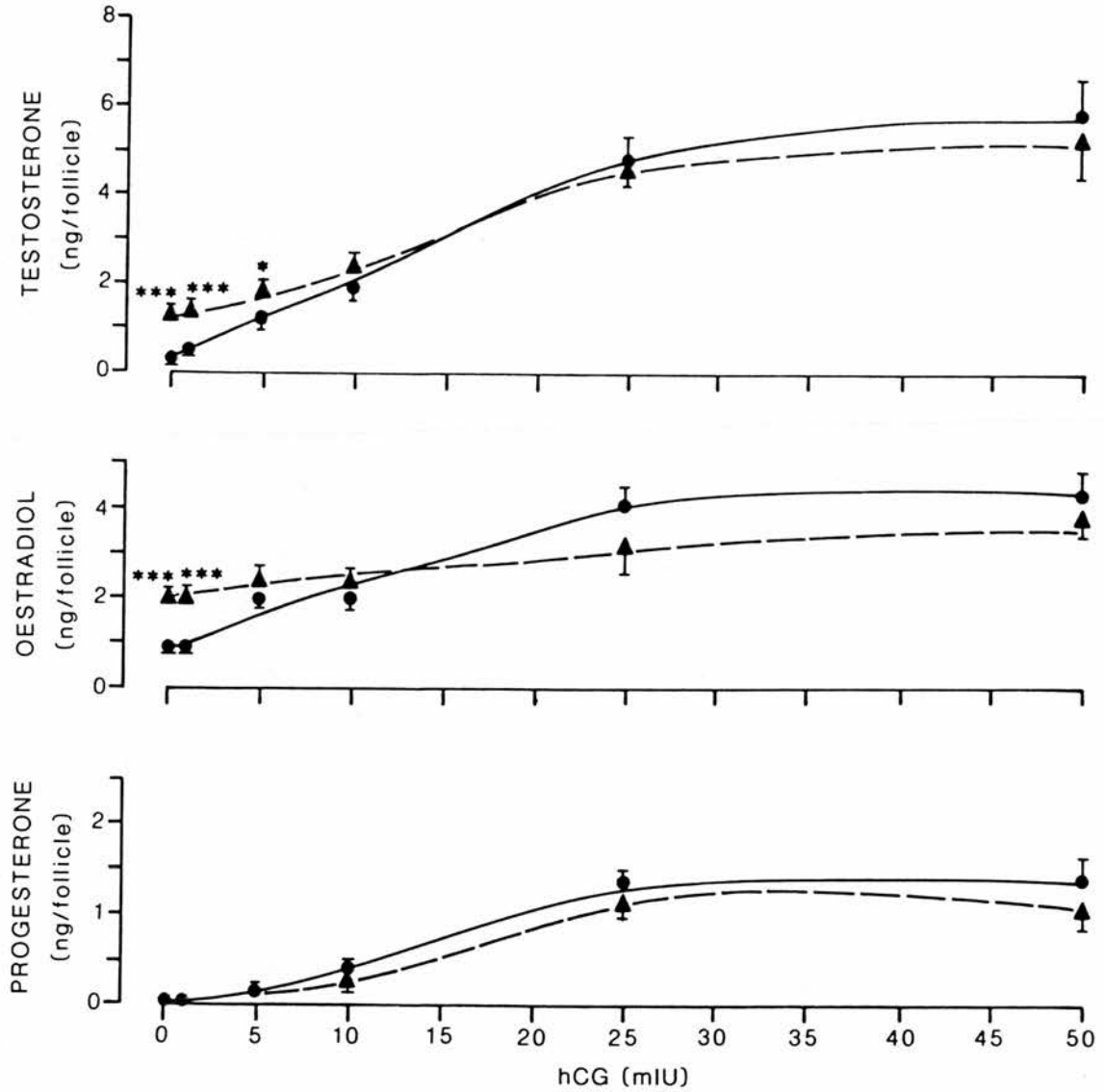

FIGURE 7.12 Stimulation of testosterone, oestradiol and progesterone release from isolated follicles incubated with various doses of hCG in the presence (▲--▲) or absence (●—●) of 10^{-8}M LHRH agonist for 3 h.

Each point represents Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; *** $P < 0.001$ for LHRH agonist treated compared to control values at each dose of hCG.

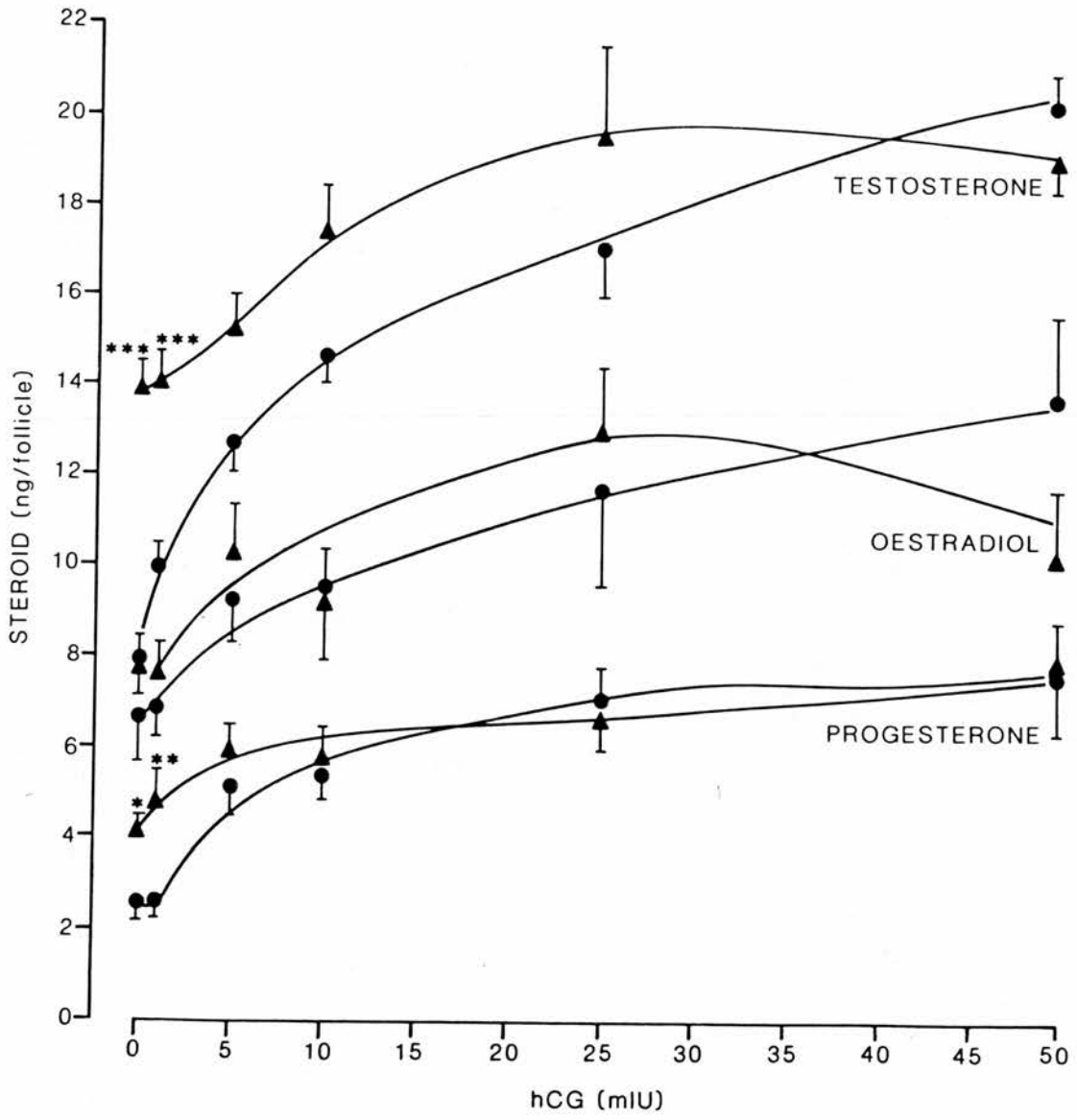

FIGURE 7.13 Stimulation of testosterone, oestradiol and progesterone production by isolated follicles incubated with various doses of hCG in the presence (▲—▲) or absence (●—●) of 10^{-8}M LHRH agonist for 17 h.

Each point represents Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for LHRH agonist treated compared to control values at each dose of hCG.

incubated for a further 16 h.

Results:- The results of steroid release over the 3 h and 17 h incubation periods are shown in Figs. 7.12 and 7.13.

Analysis of the results showed a significant effect of LHRH agonist on hCG-stimulated testosterone at 3 h ($P < 0.005$) and 17 h ($P < 0.005$). Subsequent analysis showed significant stimulation by LHRH agonist occurred with doses of 1 mIU and 5 mIU hCG at 3 h, and with 1 mIU at 17 h.

LHRH agonist however, had a significant effect on hCG-induced oestradiol production ($P < 0.05$) after 3 h but not after 17 h. LHRH agonist stimulated basal oestradiol and that in the presence of 1 mIU hCG after 3 h incubation.

LHRH agonist significantly increased hCG-induced progesterone production at 17 h ($P < 0.05$) but not at 3 h, with stimulation of basal and 1 mIU hCG induced progesterone production after 17 h incubation.

These data indicate that LHRH enhanced the sterodogenic response to low doses of hCG, due to stimulation of basal and 1 mIU hCG-stimulated steroid production. No inhibition of hCG stimulation was observed with LHRH agonist treatment.

7.7.2 Effect on time course of hCG action

In order to examine whether LHRH exerts a transitory effect on hCG-induced steroid production a detailed time course similar to that described earlier (see 7.3.5) was investigated.

Method:- Four groups of 10 follicles were incubated with medium alone, 10^{-8}M LHRH agonist, 50 mIU hCG or both 10^{-8}M LHRH agonist and 50 mIU hCG. Medium was replaced with fresh containing the same hormone additions every 2 h up to 6 h, then the follicles and medium were

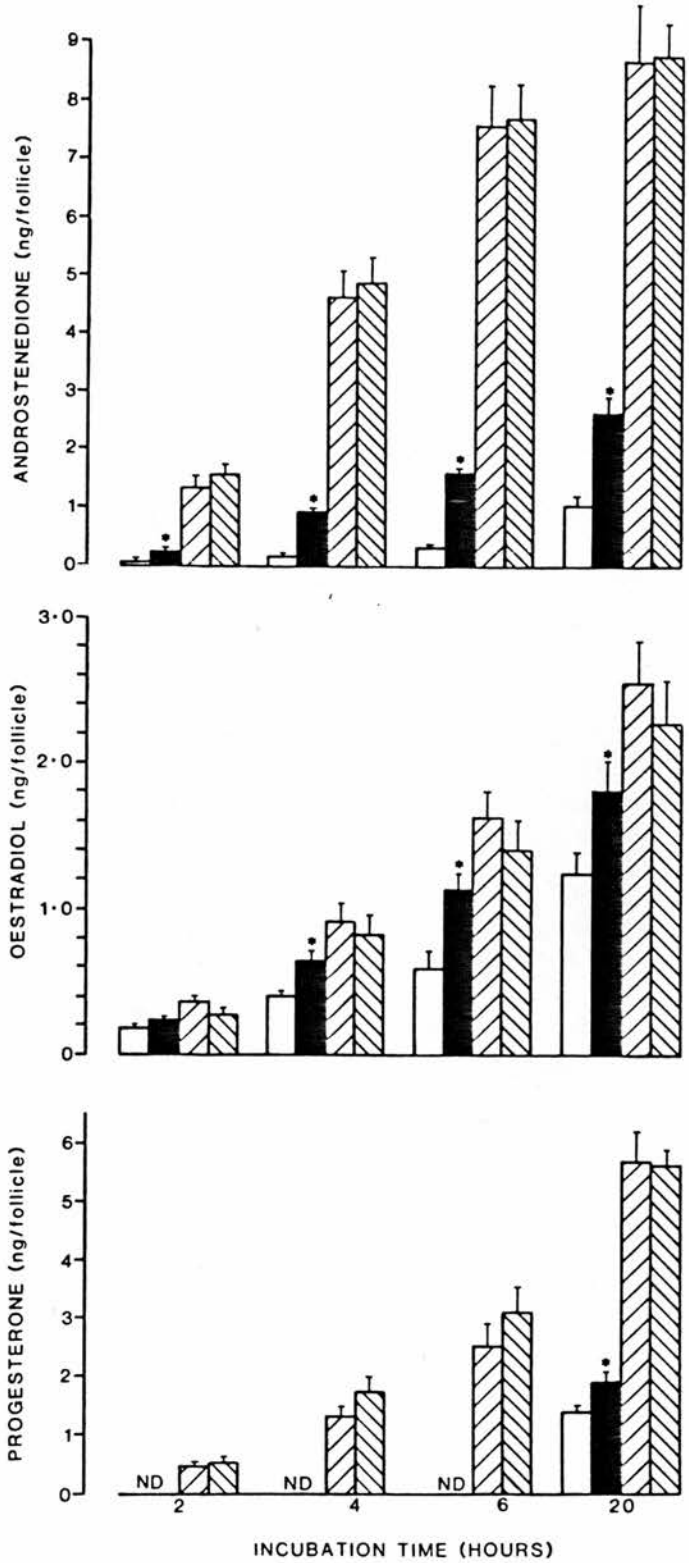

FIGURE 7.14 Time course of androstenedione, oestradiol and progesterone release from follicles incubated with medium alone (□), 10^{-8}M LHRH agonist (■), 50mIU hCG (▨) or 50mIU hCG and 10^{-8}M LHRH agonist (▧). Medium was replaced at 2, 4, 6 and 20 h.
Data represents cumulative totals (Mean ± S.E.M., 10 follicles per group) of steroid production over each time period.
ND = Non Detectable
* $P < 0.001$ for LHRH agonist treated compared to non-LHRH agonist treated values at each time period.

TABLE 7.4

Steroid release from follicles incubated with hCG or medium alone with or without 10^{-8}M LHRH agonist for 2 h periods upto 6 h and from 6-20 h.

Data are mean ± S.E.M. (10 follicles/group). Values differ from M199 controls: [a] $P < 0.001$; [c] $P < 0.05$ for LHRH agonist treated compared to non-LHRH agonist treated values at each time.

Time (h)	Steroid ng/follicle	Treatment			
		Control (M199)	10^{-8}M LHRH Agonist	50 mIU hCG	10^{-8}M LHRH Agonist 50 mIU hCG
0-2	Androstenedione	73± 8	2772± 21[a]	1349±192	1611±176
	Testosterone	231± 28	549± 48[a]	2994±389	2810±345
	Oestradiol	192± 17	230± 20	369± 36	279± 37
	Progesterone	ND	ND	426± 57	511± 80
2-4	Androstenedione	152± 19	667± 49[a]	3229±293	3271±289
	Testosterone	233± 20	992± 75[a]	7840±466	8415±734
	Oestradiol	216± 28	419± 46[a]	562± 67	496± 81
	Progesterone	ND	ND	942±122	1241±172
4-6	Androstenedione	165± 16	640± 58[a]	2914±334	2799±271
	Testosterone	343± 37	957± 77[a]	7121±598	6973±358
	Oestradiol	257± 44	496± 44[a]	700± 83	580±110
	Progesterone	ND	ND	1196±193	1389±185
6-20	Androstenedione	698±106	1542±255[a]	1165±206	1101±133
	Testosterone	2866±321	3666±411[c]	2933±322	2891±135
	Oestradiol	339±107	689± 87[a]	933±124	865±173
	Progesterone	1411±146	1840±100[c]	3159±238	2481±118

frozen at 20 h.

Results:- The cumulative pattern of androstenedione, oestradiol and progesterone release is shown in Fig. 7.14 and the steroid release per time period in Table 7.4. Similar results to those attained previously were seen with LHRH agonist or hCG alone, namely hCG stimulation of production of all steroids after 2 h incubation, and LHRH agonist stimulation of androgen production after 2 h but without effect on progesterone until 6-20 h. LHRH agonist had no effect on the steroidogenic response to 50 mIU hCG at any of the times studied (Fig. 7.14; Table 7.4).

7.7.3 Effect of Pre-incubation

Studies in luteal tissue and tissue from immature rat ovaries had indicated that pre-incubation with LHRH agonist would result in a decreased response to subsequent gonadotrophin exposure (Reddy et al., 1980; Hall et al., 1981). The following experiment set out to determine the effects of pre-incubation of isolated follicles with 10^{-8}M LHRH agonist, on the subsequent steroidogenic response to 1 IU hCG.

Method:- Four groups of 20 follicles were pre-incubated either with or without 10^{-8}M LHRH agonist for 1, 3, 6 or 18 h, before addition of 1 IU hCG.

Results:- Results for testosterone and oestradiol release are shown in Table 7.5. Testosterone release was stimulated after each pre-incubation period longer than 1 h, with LHRH agonist ($P < 0.001$). No such effect was seen for oestradiol. hCG stimulated both oestradiol and testosterone production after 1, 3 and 6 h pre-incubation. Follicles pre-incubated for 18 h either with or without LHRH agonist were unable to

TABLE 7.5

Testosterone and oestradiol production in response to preincubation with 10^{-8}M LHRH agonist for 1, 3, 6 or 18 h and after subsequent incubation with 1 IU hCG for 3 or 11 h.

Data are mean ± S.E.M. (10 follicles/group). $P < 0.001$ for LHRH agonist treated compared to non-LHRH agonist treated values at each time period.

Steroid pg/follicle	Time of preincubation	Medium after preincubation -LHRH Agonist	+LHRH Agonist	Time of exposure to hCG (1 IU)	Medium after exposure to hCG -LHRH Agonist	+LHRH Agonist
Testosterone	1 h	ND	ND	3 h	2096±141	2082±352
Oestradiol		ND	ND		977±153	982±119
Testosterone	3 h	671±148	1481± 130[a]	3 h	4193±627	5064±790
Oestradiol		383± 48	377± 101		1621±278	1921±594
Testosterone	6 h	921±105	3111± 278[a]	11 h	3586±745	5298±842
Oestradiol		558± 67	587± 83		3294±596	2216±522
Testosterone	18 h	2254±165	5026±1201[a]	3 h	No response	
Oestradiol		1812±295	1827± 457			

respond to hCG. Pre-incubation with 10^{-8}M LHRH agonist had no effect on the subsequent response to 1 IU hCG.

7.7.4 Summary

(1) LHRH agonist had no effect on steroidogenic changes induced by 50 mIU hCG over 2-20 h incubation.

(2) LHRH agonist had no inhibitory effect on tissue responsiveness to hCG. The steroid response to extremely low (1 mIU) doses of hCG was enhanced by LHRH agonist.

(3) Pre-incubation with LHRH agonist for 1-6 h had no effect on subsequent hCG-induced changes.

7.8 EFFECTS OF LHRH AGONIST ON cAMP-INDUCED STEROIDOGENESIS

7.8.1 Effect on dibutyryl cAMP-induced changes

Previous studies had indicated a variety of effects of LHRH on cAMP-induced steroidogenesis with either an inhibition (e.g. Reddy et al., 1980; Hillier et al., 1981) or no effect (e.g. Harwood et al., 1980a; Behrman et al., 1980; Jones et al., 1982) being reported. High doses ($>10^{-3}$M) of the membrane-soluble cAMP analogue dibutyryl cAMP (db cAMP) are toxic to cells, but a dose of 10^{-3}M was reported as being maximally effective in stimulating steroidogenesis (Hillier et al., 1978). The effect of LHRH on steroidogenesis induced by 1 mM db cAMP was therefore assessed.

Method:- Two groups of 20 follicles were incubated with control medium or 1 mM db cAMP in the presence or absence of 10^{-8}M LHRH agonist for 6 h, then transferred to fresh medium containing the same additions for 12 h.

Results:- Results are shown in Fig. 7.15. As expected 10^{-8}M

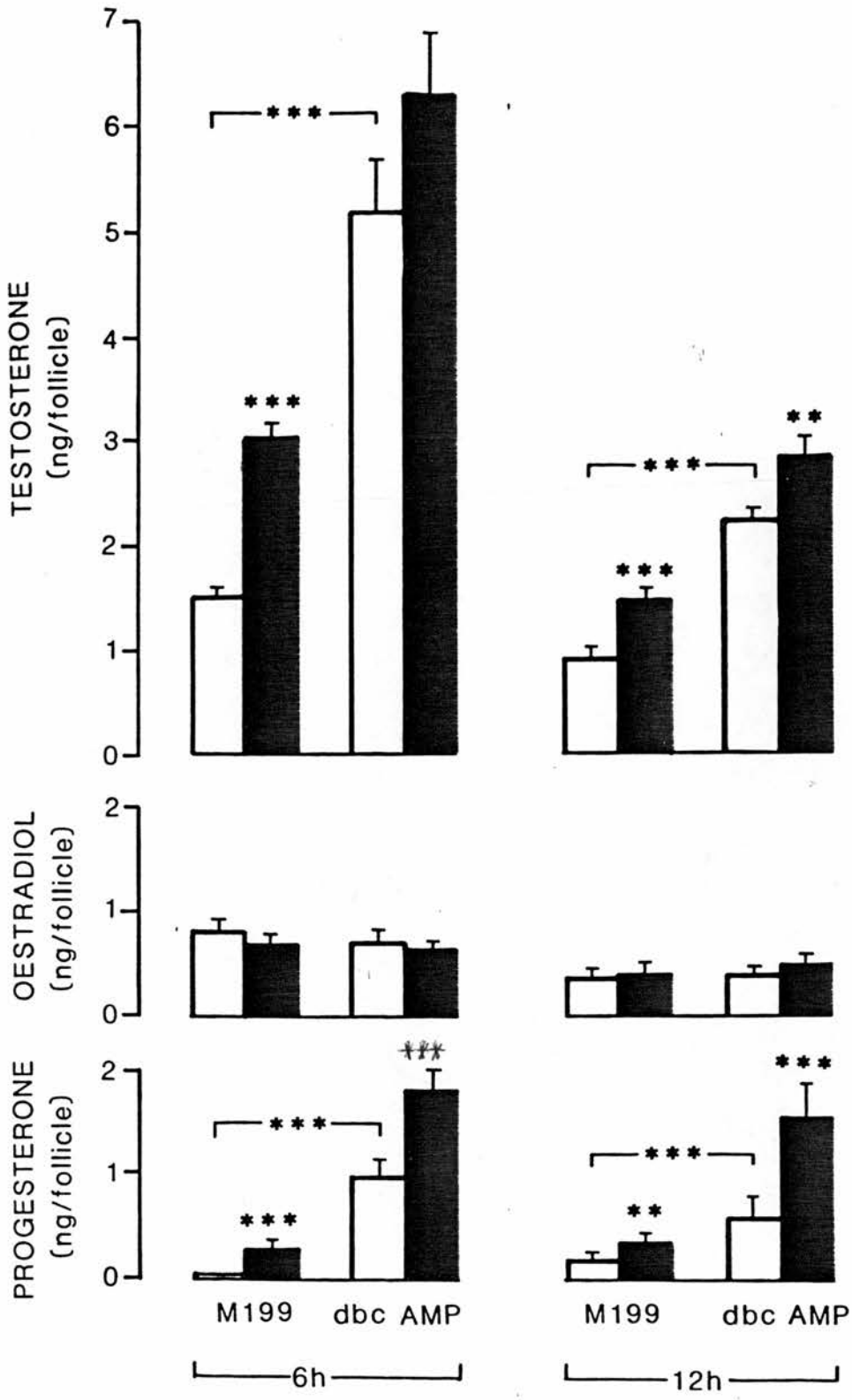

FIGURE 7.15 Testosterone, oestradiol and progesterone production by follicles incubated for 6 h then 12 h with M199 or 1 mM dibutyryl cyclic AMP (db cAMP) in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data are Mean ± S.E.M. (10 follicles per group).

** $P < 0.01$; *** $P < 0.001$ for LHRH agonist treated compared to controls in each group, or as indicated.

LHRH agonist alone stimulated basal testosterone and progesterone release ($P < 0.001$) after 6 h and 12 h. Db cAMP also stimulated the accumulation of testosterone and progesterone at both times. LHRH had no effect on the db cAMP-induced testosterone increase at 6 h but stimulated over and above that of db cAMP at 12 h ($P < 0.01$) and after both 6 h and 12 h for progesterone release ($P < 0.001$). Thus LHRH appeared to potentiate the effects of db cAMP on testosterone and progesterone release and may therefore exert stimulatory effects at events post-cAMP formation and/or involve a mechanism independent of that of cAMP.

The absence of an effect of db cAMP on oestradiol production was unexpected, particularly since testosterone was markedly increased. However, this phenomenon seems to reflect one of the major problems encountered during these studies, namely the variability of oestradiol responses. These discrepancies are unlikely to be due to differences in responses of individual animals to PMSG and it is not known why the aromatase system seems unable to respond to the presence of precursor under certain conditions but not others.

However, in complete contrast to earlier observations indicating no effect or inhibition of cAMP-induced steroidogenesis, short-term exposure of LHRH agonist to isolated rat follicles enhanced the progesterone and testosterone response to 1 mM db cAMP.

7.8.2 Effect on caffeine-induced changes

Since methyl xanthenes such as caffeine are potent inhibitors of phosphodiesterase (the enzyme responsible for cAMP breakdown), addition of such compounds would be expected to raise endogenous cAMP levels. The effect of caffeine on LHRH agonist-induced steroidogenesis was therefore

TABLE 7.6

Effect of 4 mM caffeine on LHRH agonist-stimulated steroidogenesis in isolated rat pre-ovulatory follicles.

Values are mean ± S.E.M. (10 follicles/group). [b] $P < 0.01$; [c] $P < 0.001$ for LHRH agonist treated compared to non-LHRH agonist treated values.

Steroid production (ng/follicle)	Treatment			
	M199	10^{-8}M LHRH Agonist	4 mM Caffeine	4 mM Caffeine + 10^{-8}M LHRH Agonist
Testosterone	5.1 ±0.3	7.7±0.4[c]	5.7 ±0.5	7.6 ±0.6[c]
Oestradiol	0.81±0.14	1.5±0.2[b]	0.81±0.1	1.12±0.1[c]
Progesterone	2.7 ±0.2	3.8±0.3[b]	4.7 ±1.4	6.2 ±0.6[c]

examined.

Method:- Four groups of 10 follicles were incubated with M199, 4 mM caffeine, 10^{-8}M LHRH agonist or both caffeine and LHRH agonist, for 17 h.

Results:- The results obtained are shown in Table 7.6. LHRH agonist alone stimulated both testosterone ($P < 0.001$), progesterone ($P < 0.01$) and oestradiol ($P < 0.01$) levels. Caffeine alone had no effect on basal levels of testosterone or oestradiol but increased basal progesterone production ($P < 0.001$). Caffeine had no effect on LHRH agonist-stimulated testosterone and oestradiol production. LHRH agonist stimulated progesterone production over and above that found with caffeine alone.

Discussion:- The results of the previous 2 experiments are consistent with the theory that the stimulatory actions of LHRH agonist are additional to those of cAMP, whether administered exogenously in the form of db cAMP or after raised endogenous levels induced by caffeine. Since gonadotrophic hormones are believed to exert their effects on steroidogenesis by raising endogenous cAMP (see Marsh, 1975; 1976) it was surprising that LHRH stimulated steroidogenesis over and above that seen after db cAMP or caffeine treatment but had no effect on steroidogenesis induced by high doses of hCG (see 7.7). The reason for this difference is unknown. In the absence of measurement of endogenous cAMP levels it is not possible to determine whether LHRH treatment alters cAMP. However, no change in granulosa cell cAMP content was seen after treatment with LHRH agonist despite increased progesterone release (Clark et al., 1980). In addition, no change in cAMP content was seen in isolated follicles incubated with LHRH agonist (Hillensjo et al., 1982). It is likely

therefore that LHRH agonist-induced changes in steroidogenesis are in addition to or independent from those of cAMP.

7.9 EFFECT OF LHRH AGONIST ON PROSTAGLANDIN-STIMULATED STEROIDOGENESIS

Prostaglandins have been postulated to be involved in the regulation of follicular function, with $PGF_2\alpha$ being implicated in follicular rupture and PGE_2 in luteinization (see Armstrong, 1979 for review). In addition prostaglandins are likely to be involved in the induction of ovulation, mediating the actions of LH (e.g. Dodson & Watson, 1977).

Incubation of isolated granulosa cells with 1 µg PGE_2 together with LHRH for 48 h, resulted in inhibition of PGE_2-induced oestradiol production (Hsueh et al., 1980). This experiment set out to determine the short-term effects of LHRH agonist on steroidogenesis induced by PGE_2 or $PGF_2\alpha$.

Method:- Three groups of 20 follicles were incubated with M199, 1 µg $PGF_2\alpha$, or 1 µg PGE_2 in the presence of absence of 10^{-8}M LHRH agonist for 3 h followed by 17 h.

Results:- Control incubations showed LHRH agonist stimulating basal androgen (3 h & 17 h) and progesterone (17 h) but not oestradiol production.

PGE_2: PGE_2 stimulated basal androgen, oestradiol and progesterone release after 3 h, and basal androgen and progesterone after 17 h (Fig. 7.16). LHRH agonist inhibited the PGE_2-induced increase in progesterone after 17 h incubation ($P < 0.05$) and a small, not significant decrease in oestradiol after 17 h, but had no effect on other steroids at anytime.

$PGF_2\alpha$: $PGF_2\alpha$ stimulated basal androgen production at 3 h and 17 h.

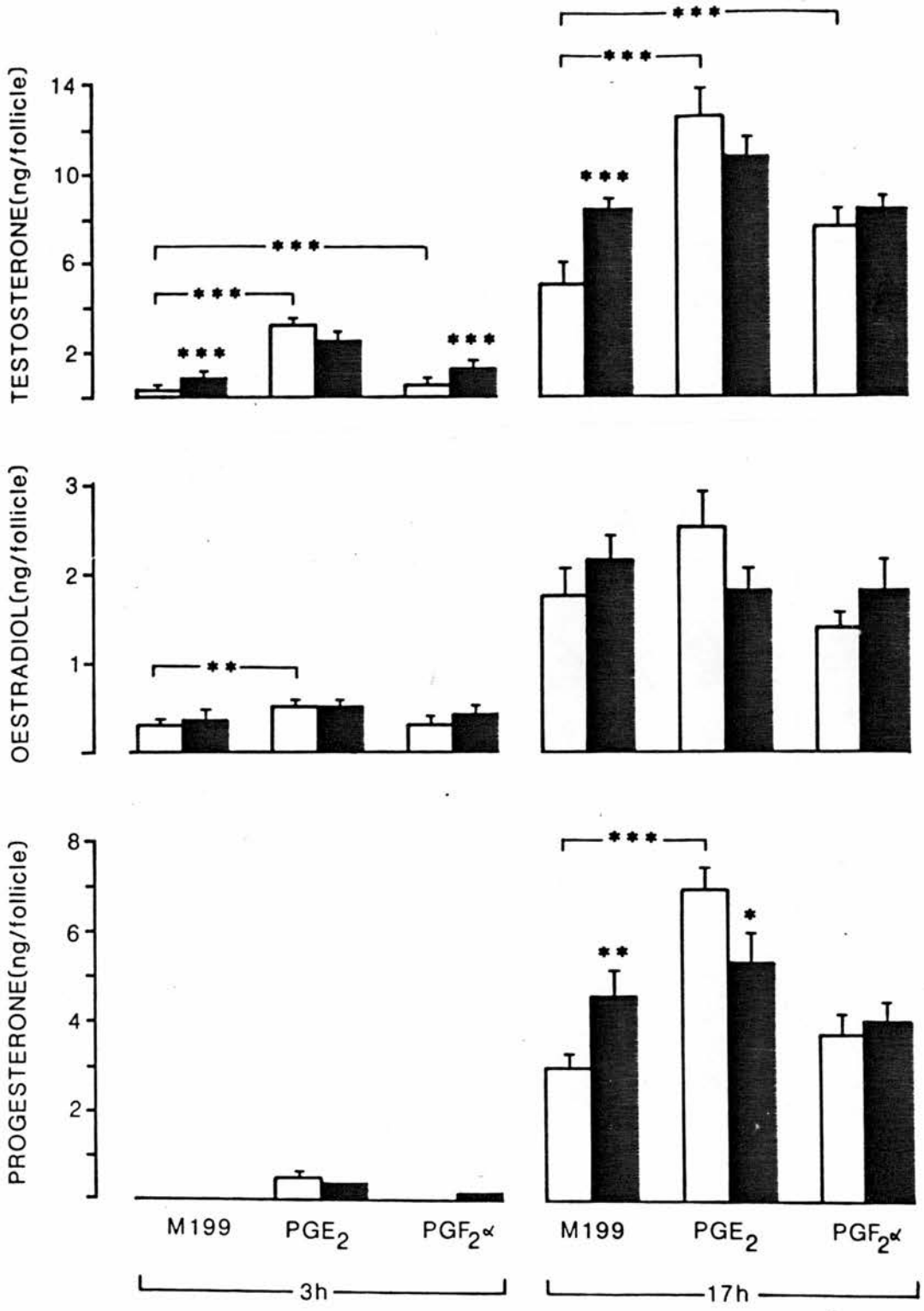

FIGURE 7.16 Testosterone, oestradiol and progesterone production by follicles incubated for 3 h then 17 h with M199, 1 μg prostaglandin $F_2\alpha$ ($PGF_2\alpha$) or prostaglandin E_2 (PGE_2) in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data are Mean ± S.E.M. (10 follicles per group).

** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for LHRH agonist treated compared to controls in each group, or as indicated.

After 3 h incubation LHRH agonist enhanced the testosterone response to $PGF_{2\alpha}$ and raised progesterone to detectable levels, but had no effect on oestradiol at any time.

These results confirm the stimulatory effect of prostaglandins on steroidogenesis and show that short-term incubation with LHRH agonist can inhibit the PGE_2-induced progesterone production, after 17 h, and stimulate $PGF_{2\alpha}$-stimulated testosterone production, after 3 h.

7.10 EFFECT OF PROSTAGLANDIN SYNTHESIS INHIBITORS ON LHRH AGONIST-STIMULATED STEROIDOGENESIS

A number of reports have indicated that LHRH stimulation of progesterone production is associated with a rise in endogenous prostaglandin production (PGE_2 & $PFG_{2\alpha}$) in isolated granulosa cells (Clark et al., 1980) and PGE_2 from isolated follicles (Hillensjo et al., 1982). LHRH has also been shown to increase the levels of ovarian PGE_2 in vivo (Ekholm et al., 1982a). Since LHRH agonist stimulated ovulation in hypophysectomized rats (Ekholm et al., 1981; Corbin & Bex, 1981) and this action was inhibited by indomethacin (Ekholm et al., 1982a; Dekel et al., 1983) it seemed likely that prostaglandins were involved in mediating the actions of LHRH agonist. The following experiment therefore set out to determine the effects of the prostaglandin synthesis inhibitors, indomethacin and aspirin, on LHRH agonist-induced stimulation of basal steroidogenesis.

7.10.1 Effect of Indomethacin

Method:- Two groups of 20 follicles were incubated with M199 or 100 uM indomethacin with or without 10^{-8}M LHRH agonist for 3 h and 17 h.

Results:- Indomethacin stimulated basal androgen production after

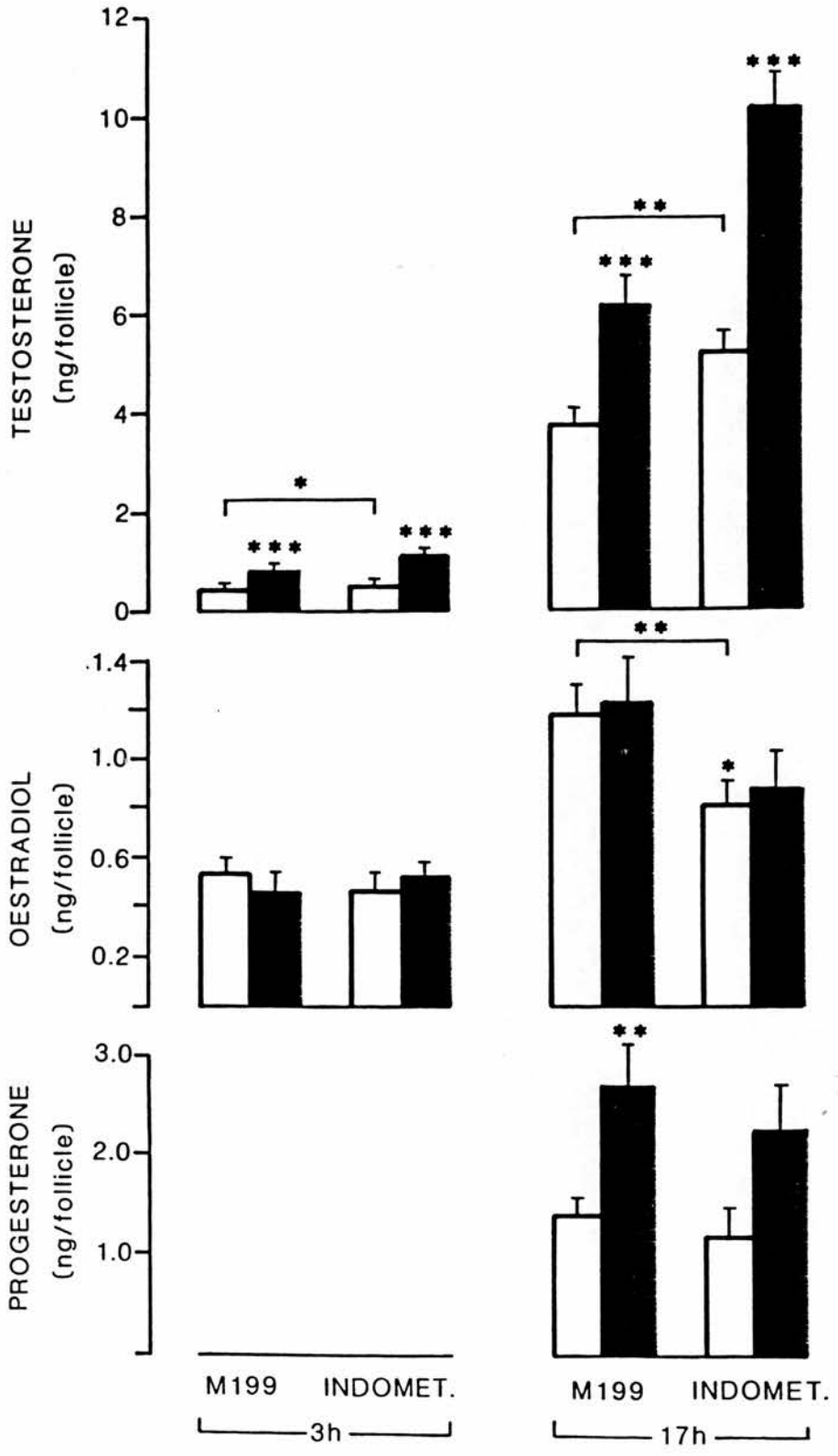

FIGURE 7.17 Testosterone, oestradiol and progesterone production by follicles incubated for 3 h then 17 h with 100 μM indomethacin (indomet) or M199 in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data represent Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3 h and 17 h and decreased basal oestradiol production after 17 h. LHRH agonist stimulated androgen production regardless of the presence of indomethacin. No effect of LHRH agonist was noted in any group, on oestradiol production. LHRH agonist-stimulated progesterone production was prevented by indomethacin (Fig. 7.17).

7.10.2 Effect of Aspirin

Method:- Two groups of 20 follicles were incubated with medium alone or 0.5 mM aspirin in the presence or absence of 10^{-8}M LHRH agonist.

Result:- Aspirin stimulated basal testosterone levels after 3 h ($P < 0.05$) and inhibited basal progesterone after 17 h ($P < 0.001$; Fig. 7.18). LHRH agonist-stimulated progesterone production was prevented by the presence of 0.5 mM aspirin. LHRH agonist-stimulated testosterone production was decreased but not prevented by aspirin (Fig. 7.18).

Discussion:- Both aspirin and indomethacin abolished the stimulatory effect of LHRH agonist on progesterone production after 17 h incubation, thus confirming the central role of endogenous prostaglandin in LHRH stimulation of granulosa cell progesterone production (Clark et al., 1980).

Interestingly, indomethacin appeared to inhibit basal aromatase and this was accompanied by an increase in basal testosterone at 17 h (Fig. 7.17). In the presence of inhibited aromatase however, LHRH agonist further stimulated testosterone production thus implicating a direct thecal site of action.

In contrast to progesterone, LHRH agonist-stimulated testosterone production occurred despite the presence of indomethacin or aspirin. It is concluded therefore that the thecal action of LHRH, unlike the

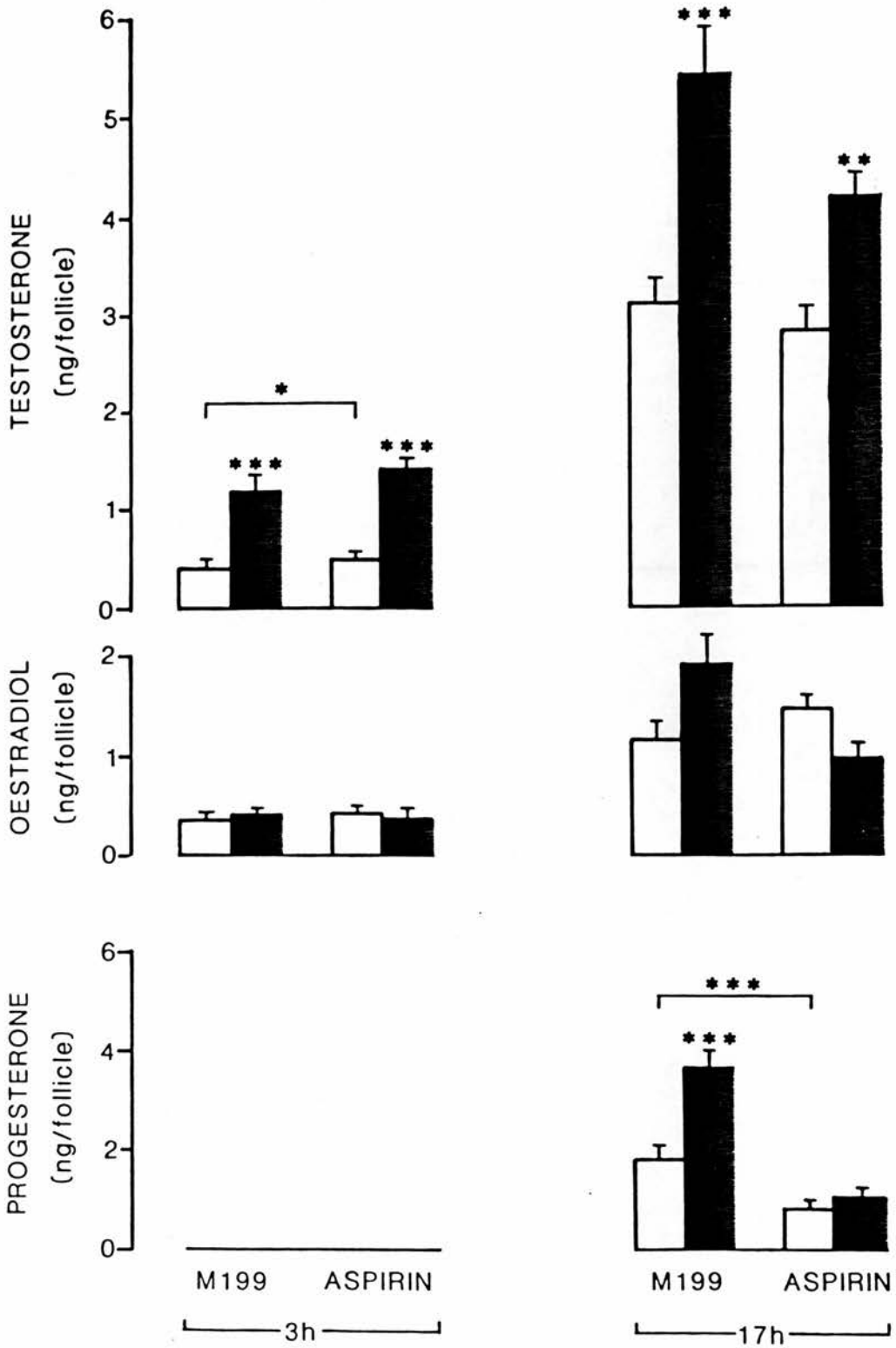

FIGURE 7.18 Testosterone, oestradiol and progesterone production by follicles incubated for 3 h then 17 h with 0.5 mM aspirin or M199 in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data represents Mean ± S.E.M. (10 follicles per group).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for LHRH agonist treated compared to controls in each group, or as indicated.

granulosa action, does not involve endogenous prostaglandin production.

7.11 SUMMARY

1) LHRH agonist stimulated testosterone and progesterone production in the presence of db cAMP or caffeine.

2) LHRH agonist inhibited PGE_2-stimulated progesterone production, but had no effect on other PGE_2-induced changes.

3) LHRH agonist stimulated testosterone production in the presence of $PGF_{2\alpha}$ after 3 h but had no effect on other steroids at any time.

4) LHRH agonist-stimulated progesterone was abolished by prostaglandin synthetase inhibitors whereas LHRH agonist-stimulated testosterone production was not abolished.

7.12 RESPONSIVENESS OF FOLLICLES TO LHRH AGONIST DURING DIFFERENT STAGES OF DEVELOPMENT

In order to assess whether follicles from different stages of development were capable of responding to LHRH agonist, the responsiveness of follicles isolated from ovaries from immature untreated rats was assessed together with that of follicles isolated from ovaries from immature rats 24 h after exposure to 4 IU PMSG.

7.12.1 "Immature" follicles

Method:- Two groups of 20 follicles were isolated from immature rat ovaries and incubated with M199 or 50 mIU hCG in the presence or absence of 10^{-8}M LHRH agonist, for 17 h.

Results:- The follicles isolated were small, approximately half (i.e. 0.5 - 0.8 mm) the diameter of those recovered 48 h after PMSG treatment. Basal androstenedione and oestradiol levels were non-detectable, the major steroid produced being progesterone. 50 mIU hCG

TABLE 7.7

Steroid production from follicles isolated from immature rat ovaries incubated for 17 h.

Values are mean ± S.E.M. (10 follicles/group); ND = Non-detectable.

Treatment	Steroid pg/follicle		
	Androstenedione	Oestradiol	Progesterone
M199	ND	ND	1201±439
10^{-8}M LHRH agonist	ND	ND	1535±343
50 mIU hCG	116±13	ND	4600±928
50 mIU hCG + 10^{-8}M LHRH agonist	90±17	ND	4325±796

stimulated progesterone production and raised androgen to detectable levels.

LHRH agonist had no effect either on basal or hCG-stimulated steroidogenesis (Table 7.7).

7.12.2 Dioestrous-type follicles

Method:- Two groups of 20 follicles were isolated from rat ovaries 24 h after treatment with 4 IU PMSG. Follicles were incubated with M199 or 50 mIU hCG in the presence or absence of 10^{-8}M LHRH agonist, for 17 h.

Results:- Follicles were slightly smaller (approximately ¾ size) than those isolated 48 h after PMSG (i.e. 0.7 - 0.9 mm diameter). The steroidogenic response of these follicles was similar to that of pro-oestrous follicles. LHRH agonist significantly increased basal production of androstenedione, oestradiol and testosterone ($P < 0.01$; Fig. 7.19) but had no effect on hCG-induced steroidogenesis (Fig. 7.19).

7.13 DISCUSSION

In contrast to previous observations showing inhibitory actions of LHRH and LHRH agonist on ovarian function (see Chapter 1) the data presented in this chapter have indicated that the short-term effects of LHRH and LHRH agonist on ovarian function are stimulatory as regards progesterone, testosterone and occasionally, oestradiol production. Other investigators have confirmed the stimulatory actions of LHRH agonist on granulosa cell progesterone production both *in vitro* (Clark *et al.*, 1980; Hillensjo *et al.*, 1982) and *in vivo* (Ekholm *et al.*, 1981). In addition LHRH has been found to stimulate a variety of parameters such as prostaglandin accumulation (Clark *et al.*, 1980), meiotic maturation of

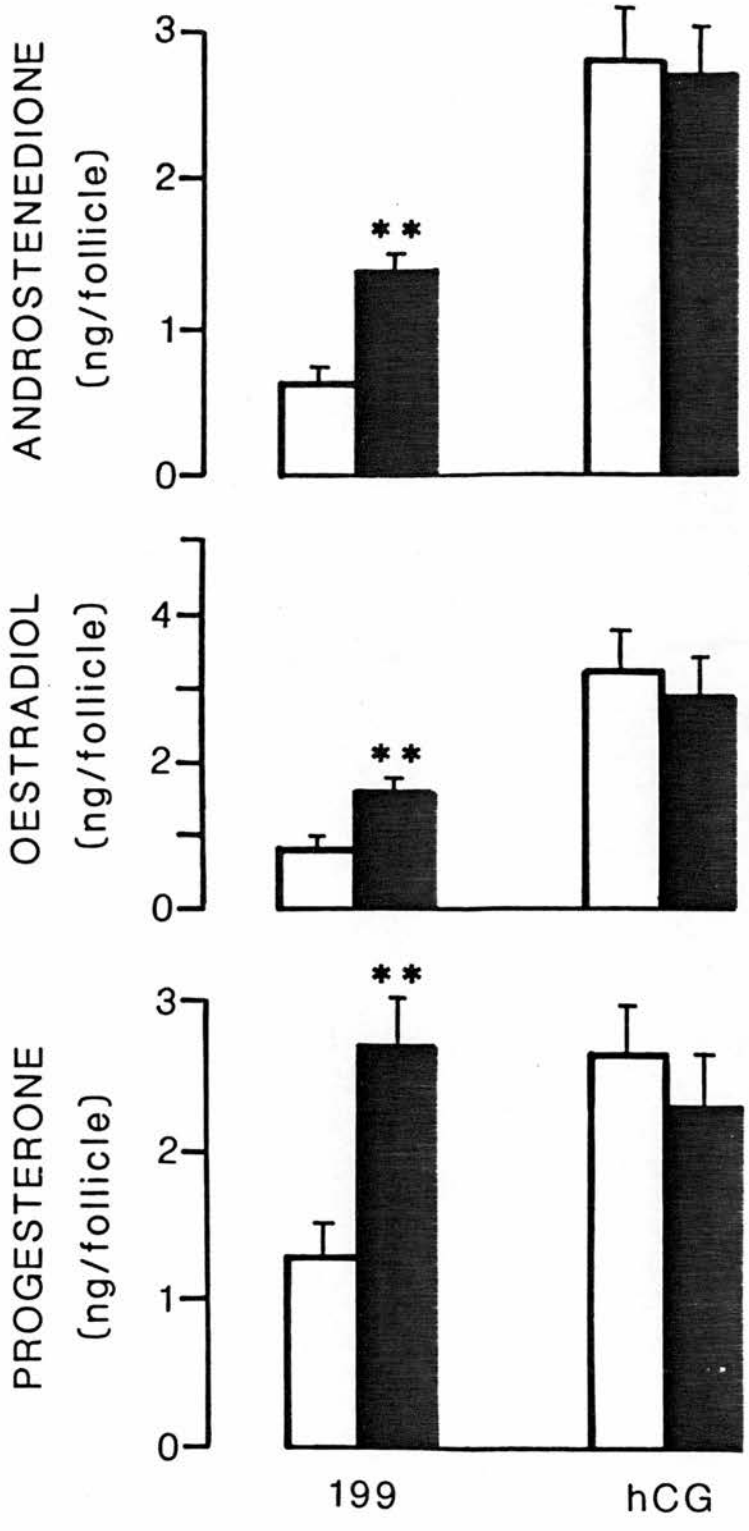

FIGURE 7.19 Androstenedione, oestradiol and progesterone production from follicles isolated from rats 24 h after treatment with PMSG. Follicles were incubated with or without 50 mIU hCG in the presence (■) or absence (□) of 10^{-8}M LHRH agonist.

Data are Mean ± S.E.M. (10 follicles per group).

** $P < 0.01$ for LHRH agonist treated compared to control values in each group.

oocytes *in vitro* and *in vivo* (Hillensjö & LeMaire, 1980; Ekholm *et al.*, 1981; 1982a) and oocyte respiration (Magnusson & LeMaire, 1981). LHRH and LHRH agonists have even been reported to induce ovulation in hypophysectomised pro-oestrous rats (Corbin & Bex, 1981; Ekholm *et al.*, 1981). In these actions LHRH appears to mimic the effects of LH; however LHRH does not mediate the action of LH since LHRH antagonists inhibited LHRH but not LH-induced oocyte maturation in isolated follicles (Ekholm *et al.*, 1982b; Dekel *et al.*, 1983). Since LHRH has no effect on maturation of non-follicle enclosed isolated oocytes (Anderson & Hillensjö, 1982; Hillensjö *et al.*, 1983), its effects on oocyte maturation are likely to be secondary to the induced changes in follicular environment and/or to its interruption of oocyte-corona cell contact (Erickson *et al.*, 1983).

Studies in the male have indicated that short-term incubation with LHRH and LHRH agonist stimulated basal testosterone production *in vitro* (Sharpe & Cooper, 1982b; Hunter *et al.*, 1982) and *in vivo* (Sharpe *et al.*, 1982b).

The stimulatory effects of LHRH agonist are small in comparison to those of maximally or sub-maximally stimulating doses of hCG/LH. In addition LHRH had no effect on the steroidogenic response to 10 mIU - 1 IU hCG. This agrees with data of others, where LH-induced progesterone was unaffected by LHRH agonist (Clark, 1982). However, since LHRH agonist stimulated androgen production in the presence of low (1-5 mIU) hCG it may be suggested that an endogenous LHRH-like factor could be involved in the maintenance of steroid levels in the presence of low circulating levels of gonadotrophin. Moreover, studies in the male have indicated that LHRH agonist enhances the testosterone response to low doses of hCG *in vitro*

(Sharpe & Cooper, 1982b; Hunter *et al.*, 1982) and *in vivo* (Sharpe *et al.*, 1982b).

It is important to remember, however, that the isolated follicles utilized in these studies have been exposed to gonadotrophins *in vivo*. Since follicles incubated in medium alone continued to synthesize steroids it could be suggested that this was due to residual gonadotrophin stimulation and therefore that the term 'basal' steroidogenesis is a misnomer; steroid production being effected by previous gonadotrophin exposure.

That the actions of LHRH may be dependent upon prior exposure to gonadotrophins is indicated by the preliminary data presented on the responsiveness of 'immature' and 'dioestrous-type' follicles. LHRH agonist had no direct stimulatory effects on the former follicles, whereas marked stimulation was seen with follicles exposed for 24 or 48 h to PMSG. Recent studies have indicated that in the male, LH regulates the responsiveness of Leydig cells to LHRH agonist (Sharpe & Fraser, 1983); it would be interesting to determine whether a similar phenomenon occurs in the female i.e. if pre-exposure to LH determines the sensitivity of the follicle to LHRH stimulation.

The mechanism of LHRH action on ovarian tissue is largely unknown. Its stimulation of progesterone is unlikely to involve cAMP (Clark *et al.*,1980; Hillensjö *et al.*, 1982). It appears well established that LHRH agonist-induced ovulation, like that of LH, is mediated by increased ovarian prostaglandins (e.g. Ekholm *et al.*, 1982a). In addition LHRH has been found to stimulate production of plasminogen activator, the enzyme involved in follicular rupture (Wang, 1983). From the data presented in

this chapter it appears that two separate mechanisms of LHRH action mediate the effects on granulosa and thecal function. Thus LHRH agonist-stimulated progesterone but not androgen production, was abolished by prostaglandin synthesis inhibitors, indicating a role for endogenous prostaglandins in LHRH agonist-stimulated granulosa but not thecal function.

Recent studies however, have implicated a role for calcium in LHRH stimulation. The mechanism by which LHRH stimulates pituitary function is largely believed to be via alterations in cellular calcium metabolism (see Conn et al., 1981b for review). Calcium has also been implicated in the direct gonadal effects of LHRH (e.g. Ranta et al., 1983). In addition inhibition of calmodulin has been reported to abolish the stimulatory effects of LHRH agonist on granulosa cell function (Hillensjö et al., unpublished observations cited in Rani et al., 1983).

Recent studies have indicated that the initial cellular response following LHRH binding is an incorporation of unlabelled phosphate into phosphatidylinositol (PI) in both pituitary (Snyder & Bleasdale, 1982; Raymond et al., 1982) and ovary (Naor & Yavin, 1982; Leung et al., 1983; Davis & Clark, 1983). The importance of these observations is the suggested coupling of PI metabolism with that of cellular calcium (see Putney, 1981; Berridge, 1981 for reviews). A cyclic nucleotide-independent calcium- and phospholipid-dependent protein kinase exists which is selectively activated by calcium and phospholipid (Kishimoto et al., 1980) and such an enzyme has been found in ovarian tissue (Davis & Clark, 1982). The phosphatidic acid formed after PI breakdown may

therefore acts as an endogenous calcium ionophore under neurohumoral control (Putney, 1981) thus mediating the action of LHRH. Further studies, particularly as regards thecal cells, are awaited with interest.

In conclusion therefore, the data presented in this chapter suggest that the initial response of follicles to LHRH involves stimulation of basal steroidogenesis. It could be suggested therefore that follicular responsiveness to LHRH is biphasic, with short-term exposure leading to stimulation, and more prolonged exposure resulting in desensitization and an inhibitory response. This has been suggested by studies described by Rani et al., (1983) when, after 8 h continuous exposure to LHRH, the steroidogenic response as regards androgen and oestradiol production, was abolished. In addition this has been suggested for LHRH-stimulated progesterone production from isolated follicles (see Rani et al., 1983). The short-term actions of LHRH are likely therefore to mimic those of an intra-ovarian peptide involved in the regulation of ovarian function as suggested by Ying & Guillemin, (1979). The following chapter is concerned with attempts at isolation of such a peptide.

7.14 CONCLUSIONS

1) LHRH and LHRH agonist stimulate basal steroidogenesis.
2) LHRH agonist does not influence tissue responsiveness to hCG.
3) The mechanism of LHRH agonist action is unlikely to involve cAMP.
4) LHRH agonist-stimulated progesterone production is likely to involve endogenous prostaglandin production.
5) LHRH agonist-stimulated androgen production is likely to be independent of endogenous prostaglandin production.

6) Follicles isolated from immature rat ovaries do not respond to LHRH agonist.

7) Follicles isolated 24 h after PMSG treatment show similar stimulatory effects of LHRH to those seen in pre-ovulatory follicles.

8) The responsiveness of the ovary to LHRH may well be determined therefore by prior gonadotropin exposure.

CHAPTER 8

ATTEMPTS AT ISOLATING OVARIAN LHRH-LIKE MATERIAL

CHAPTER 8

ATTEMPTS AT ISOLATING OVARIAN LHRH-LIKE MATERIAL

8.1 INTRODUCTION

8.2 AIMS

8.3 ASSAY METHODS

- 8.3.1 Radioreceptor assay
- 8.3.2 Radioimmunoassay
- 8.3.3 Sources of Tissue

8.4 EXTRACTION METHODS, RESULTS AND PROBLEMS

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8.5 DISCUSSION

8.6 CONCLUSIONS AND FUTURE PROSPECTS

8.1 INTRODUCTION

As discussed in Chapter 1 LHRH exerts direct actions on a variety of extrapituitary sites. Since LHRH is rapidly degraded (e.g. Jeffcoate et al., 1974) hypothalamic LHRH is unlikely to reach these sites in high enough concentrations. It seemed likely therefore that an LHRH-like material might be produced in extrahypothalamic areas. That this is indeed the case has been shown by techniques utilizing specific antisera for LHRH. Immunoreactive LHRH has been demonstrated by immunohistochemistry in the pineal (Wheaton, 1980; Piekut & Knigge, 1982), pancreas (Seppälä et al., 1979) mammary tumours (Seppälä & Wahlström, 1980), placenta (Khodr & Siler-Khodr, 1978b) and testis (Paull et al., 1981).

Similarly, extraction of tissues and biological fluids for assay in LHRH radioimmunoassays has revealed the presence of LHRH-like activity in milk (Amarant et al., 1982) and urine (Rettig et al., 1982). In addition a macromolecule cross reacting with an LHRH antiserum has been localized in extracts of rat liver, kidney, spleen and muscle (Barnea & Porter, 1975).

The first indication that the ovary contained an LHRH-like substance was reported in 1979. During bioassay tests for the presence of inhibin-like activity in follicular fluid a contaminating 'stimulin' was found which stimulated LH release from isolated pituitary cells (de Jong et al., 1979).

In addition, these observations were confirmed by Ying & Guillemin who coined the phrase "gonadocrinin" for an LH-releasing factor present in ovarian follicular fluid (Ying & Guillemin, 1979b;1980). These studies were subsequently extended with the reported isolation of a 3500 dalton peptide from ovarian tissue with LHRH bioactivity but with immunological

characteristics different from those of LHRH (Ying et al., 1981).

Similar evidence was emerging from studies in the male. Acid-ethanol extracts of rat interstitial fluid not only displaced binding of LHRH agonist in the rat Leydig cell radioreceptor assay, but also stimulated LH release from hemipituitaries in vitro (Sharpe & Fraser, 1980b; Sharpe et al., 1981). In addition this substance was immunochemically distinct from LHRH (Sharpe et al., 1982a; Fraser et al., 1982; Sharpe & Harmer, 1983) being recognized specifically by antiserum R103. This antiserum was one of five raised against D-Ser-But^{6} des Gly^{10} ethylamide LHRH and recognized a substance in testicular extracts, not recognized by LHRH antisera (Sharpe et al., 1981).

8.2. AIMS

The studies described in this Chapter were designed to investigate:-

1) The optimum method for extracting LHRH-like activity from ovarian tissue from a variety of sources.

2) The characteristics of such a factor as determined by radioreceptor and radioimmunoassay.

8.3 ASSAY METHODS

Prior to assay, the pH of final extract was checked and if necessary adjusted to pH 7.4.

8.3.1 Radioreceptor assay (RRA)

The ability of LHRH and LHRH agonist, but not unrelated molecules, to displace binding of ^{125}I-LHRH agonist from ovarian tissue (Chapter 3) provided the basis for an RRA for the detection of LHRH-like factors. Extracts of ovarian and control tissue were therefore tested for their ability to displace binding in the rat ovarian RRA. The degree of parallelism between the displacement of binding induced by the extract and

that induced by unlabelled LHRH agonist, providing a measure of the similarity between the molecules in their receptor interactions.

8.3.2 Radioimmunoassay (RIA)

Three RIAs were selected to analyse the immunological characteristics of the extracted material: (i) an assay specific for LHRH based on an antiserum recognizing both ends of the LHRH molecule (i.e. "conformational" antiserum) as described by Nett et al., (1973); (ii) an assay specific for the C terminal sequence of LHRH based on antiserum RO (Jeffcoate et al., 1976); (iii) an assay specific for LHRH agonist (D-Ser, Bu^{t6} des Gly^{10} ethylamide LHRH) based on antiserum R103 (Fraser et al., 1983a).

8.3.3 Sources of tissue

Bovine and porcine follicular fluids were aspirated from follicles obtained from ovaries from the local abbatoir. Ovaries were kept on ice and arrived between 1-6 h after removal from the animal.

Human follicular fluid was obtained from patients undergoing oocyte aspiration for the IVF programme and was kindly supplied by Dr. O. Djahnbakhch with the informed consent of the patient. After immediate transportation to the laboratory the fluid was placed on ice prior to extraction.

Rat tissue was obtained from Sprague Dawley rats from the laboratory colony, either from immature (26 day old) rats or from immature rats killed 48 h after induction of follicular development with 50 IU PMSG. Tissue was extracted immediately after removal from the animal (within 2-5 minutes after death by CO_2 asphyxiation).

Control Serum was either of human origin (pooled from a number of normal patients) or bovine origin (from a castrate bull). Serum was

stored in 20 ml aliquots at -20°C prior to extraction.

8.4 EXTRACTION METHODS, RESULTS AND PROBLEMS

8.4.1 Acid-ethanol extraction

A method similar to that used to extract hypothalamic LHRH (Wheaton & McCann, 1976) and testicular LHRH-like activity (Sharpe & Fraser, 1980b) was used. Preliminary tests indicated that the following procedures would recover 70-75% of ^{125}I-LHRH agonist added to serum samples.

Biological fluids from a variety of sources were extracted using an excess (10-fold) of cold 0.1 N acetic acid/ethanol solution (1:40 v/v) at -20°C. After thorough mixing at -20°C for at least 30 minutes the precipitated proteins were removed by centrifugation, at 2500 rpm for 30 minutes at 4°C. The ethanol supernatant was decanted off and evaporated to dryness using a rotary evaporator. The residue was taken up in the appropriate assay buffer (10mM Tris HCl for RRA, 0.1% BSA PBS for RIA, or distilled water if further extraction was involved) to a final concentration 3 times (Human follicular fluid) or 6 times (bovine, porcine follicular fluid and serum) that of the original volume.

Results:- Bovine follicular fluid aspirated from large ($>$ 0.5 cm diameter) or small ($<$ 0.5 cm diameter) follicles, extracted as described displaced binding of ^{125}I-LHRH agonist in the rat ovarian RRA (Fig. 8.1). Bovine serum also displaced binding.

Similarly extracted human follicular fluid and human serum also displaced binding in the rat RRA (Fig. 8.2).

Problem:- Apparent LHRH-like activity was present in serum controls as well as follicular fluid extracts.

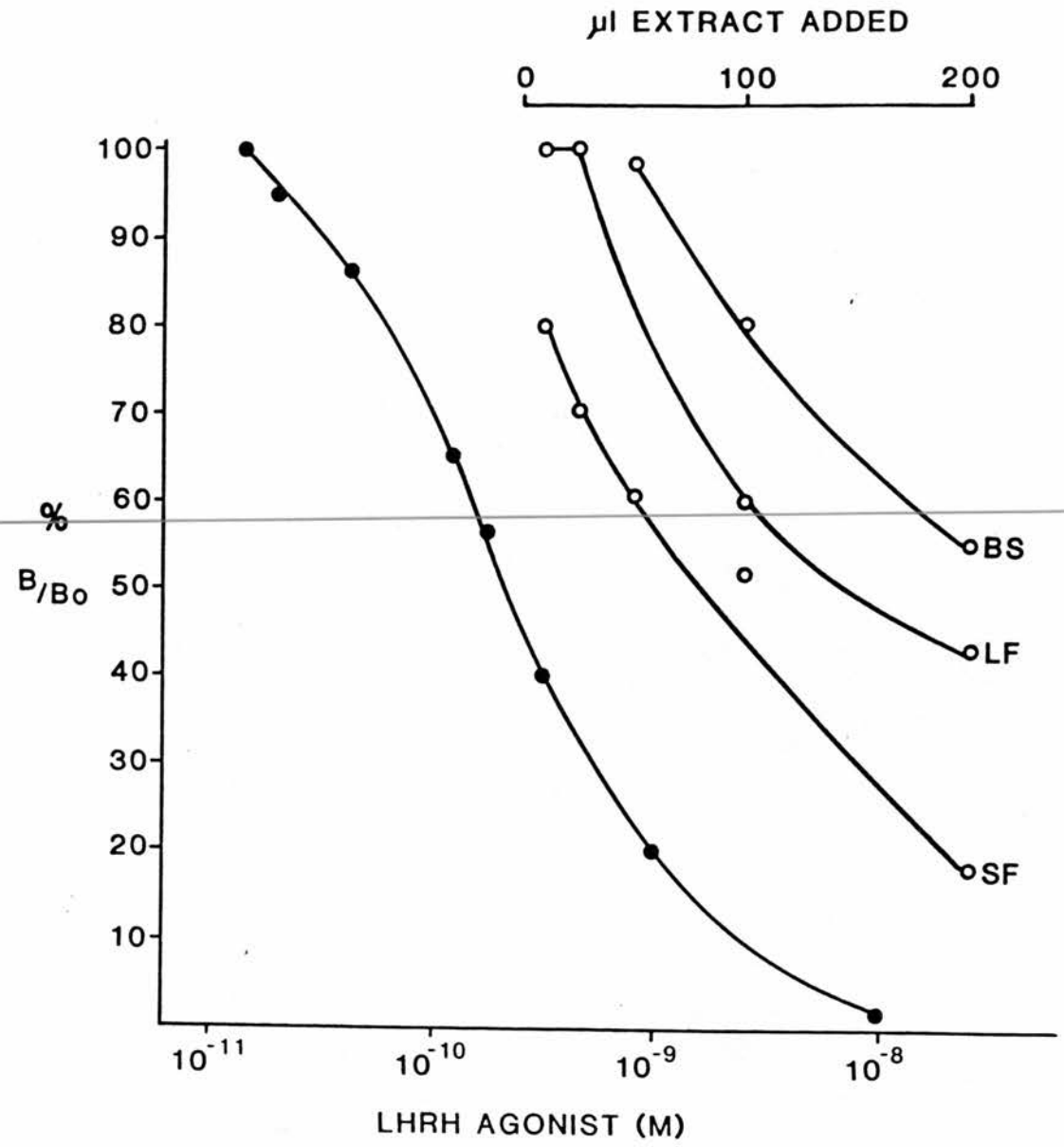

FIGURE 8.1 Displacement of ^{125}I-LHRH agonist binding to rat ovarian tissue by LHRH agonist (●—●), by acid-ethanol extracts (o—o) of bovine follicular fluid from small (SF) or large (LF) follicles, or by bovine serum (B.S.).

Each point represents mean of duplicate observations.

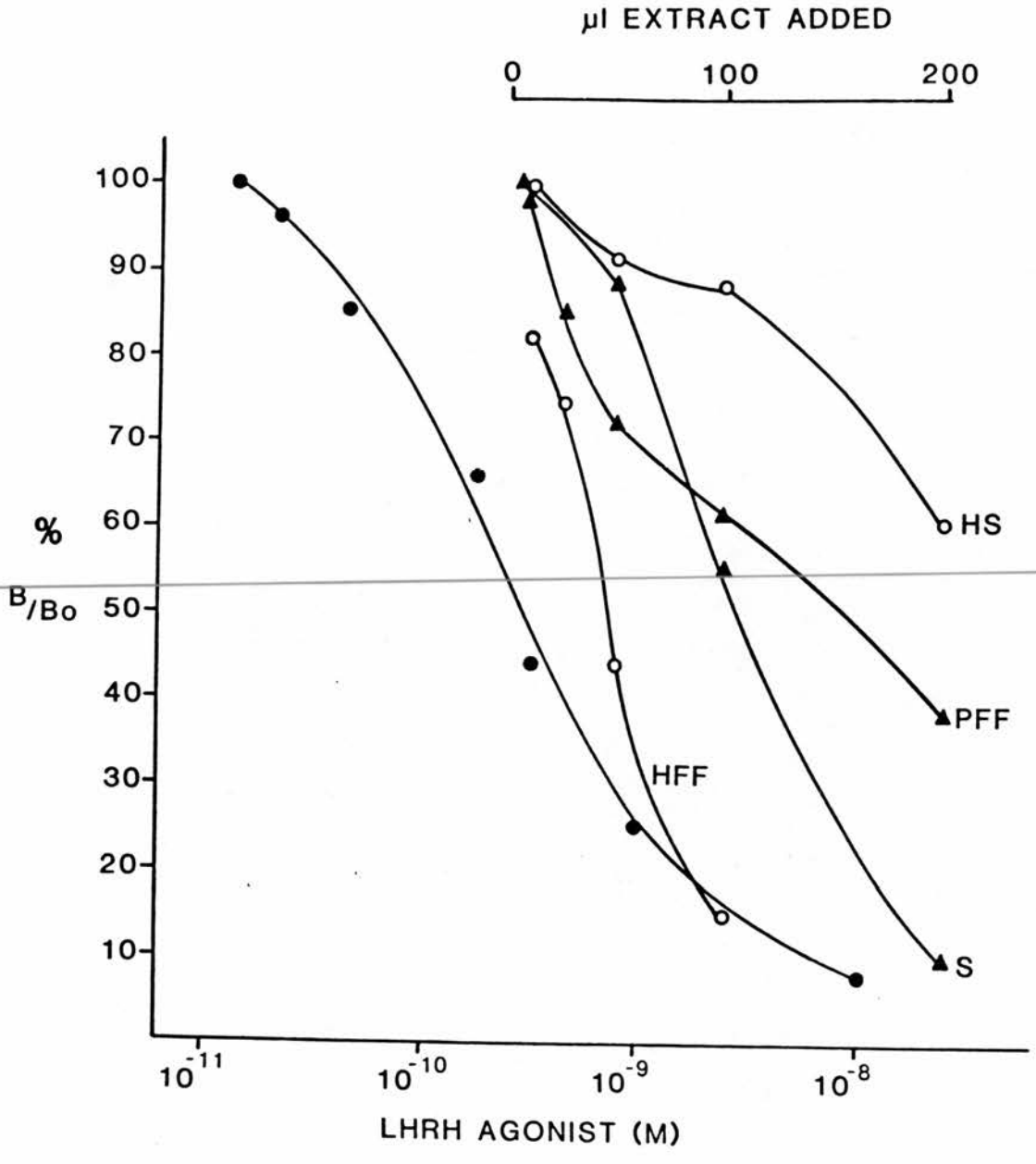

FIGURE 8.2 Displacement of ^{125}I-LHRH agonist binding to rat ovarian tissue by LHRH agonist (●—●), by acid-ethanol extracts (o—o) of human follicular fluid (HFF), and human serum (HS) or after ethyl ether acetate and but-an-1-ol extracted (▲—▲) porcine follicular fluid (PFF) and serum (S).

Each point represents mean of duplicate observations.

8.4.2. Acid-ethanol followed by ethyl ether acetate (EEA) and butan-1-ol extraction

In an attempt to eliminate non-specific interference, a fat extraction and a crude desalting step were included after the acid/ethanol procedure. After taking up the residue, left after evaporation of the ethanol, in 2 ml distilled water, samples were vortexed with 2 mls EEA. The organic layer was discarded and the aqueous layer extracted using an equal volume of butan-1-ol to dissolve peptides and leave ionic salts in the aqueous phase. The organic layer was decanted, taken to dryness under nitrogen and resuspended in assay buffer. These procedures led to the recovery of 44% of added ^{125}I-LHRH agonist tracer.

Results:- Porcine follicular fluid (from random sized follicles) and serum were extracted to final concentration 6 times the original volume. Both follicular fluid and serum displaced binding in the RRA (Fig. 8.2).

Problems:- 1) Low recovery of ^{125}I-LHRH agonist due to multiple extraction steps.

2) Serum extracts also displaced binding.

8.4.3 Amicon ultrafiltration

In a further attempt to remove potential salt interference within the assay systems a selective molecular separation technique was used. Since Diaflo UM05 filters have a molecular weight cut-off of 500 daltons, LHRH-like molecules should thus be retained while salts should be gradually diluted out via continuous dilution of the solution with fresh solvent washing the permeating species through the membrane.

The filters were washed prior to use with distilled water, to remove glycerin used in storage, and soaked overnight in 5% BSA to reduce non-specific adsorption of peptides to the filter, before being applied to

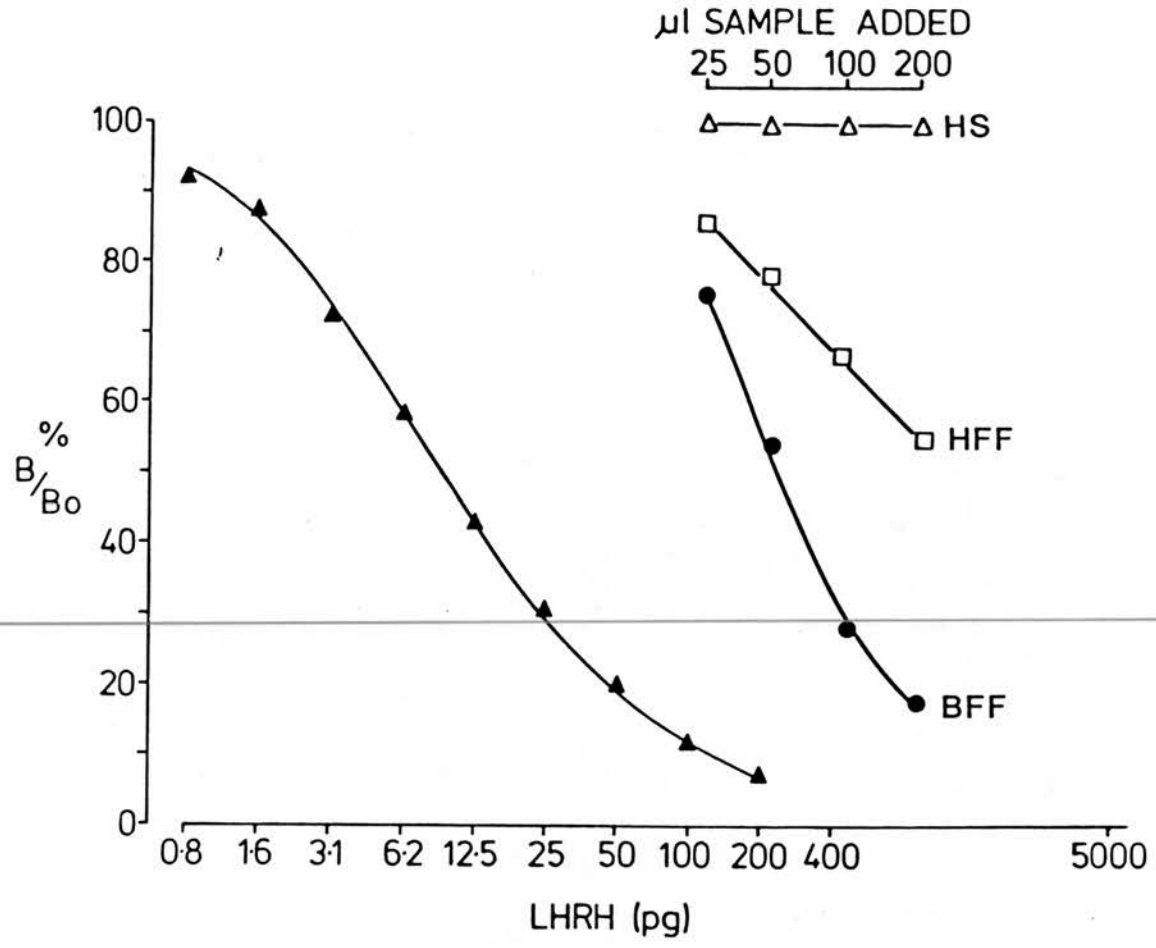

FIGURE 8.3 Cross reactivity of LHRH (▲—▲) and acid-ethanol extracts of human follicular fluid (HFF), bovine follicular fluid (BFF) and human serum (HS) after Amicon ultrafiltration, in an RIA based on antiserum RO.

Each point represents mean of duplicate determinations.

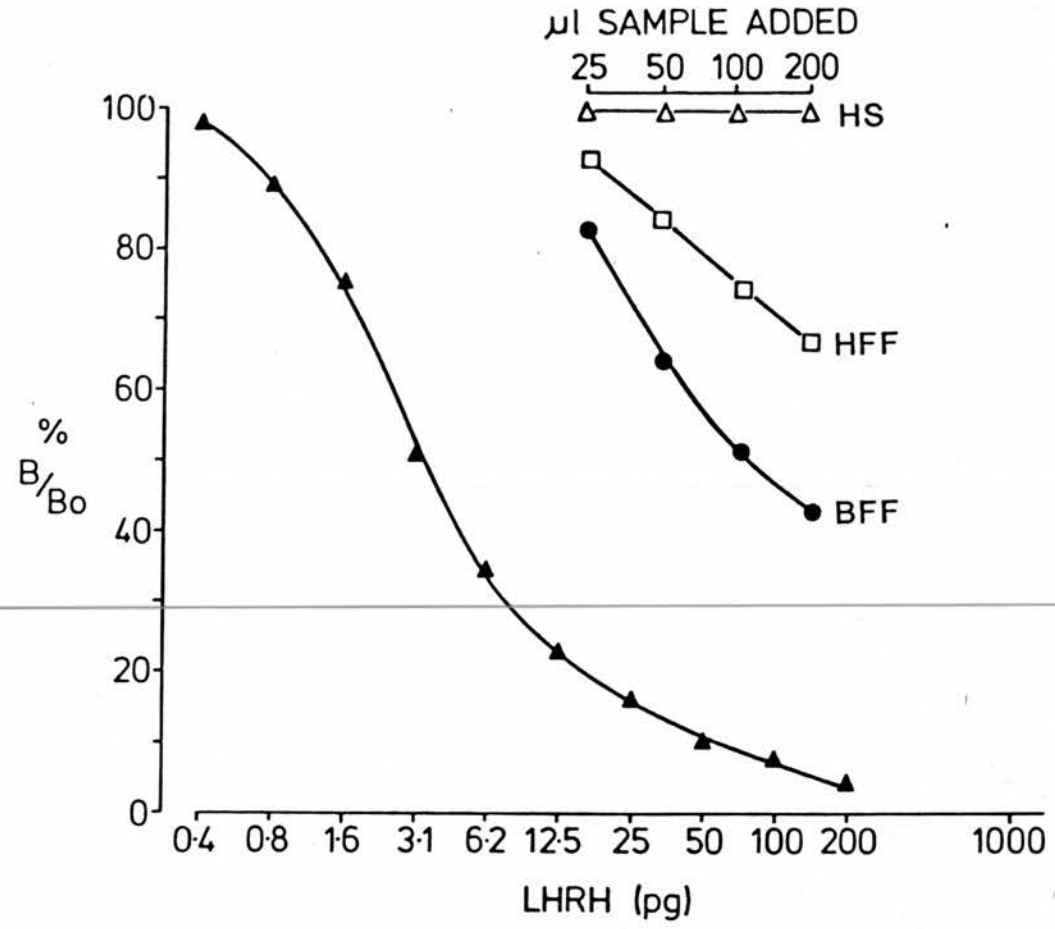

FIGURE 8.4 Cross reactivity of LHRH (▲—▲) and acid-ethanol extracts of human follicular fluid (HFF), bovine follicular fluid (BFF) and human serum (HS) after Amicon ultrafiltration, in an RIA based on the Nett antiserum.

Each point represents mean of duplicate determinations.

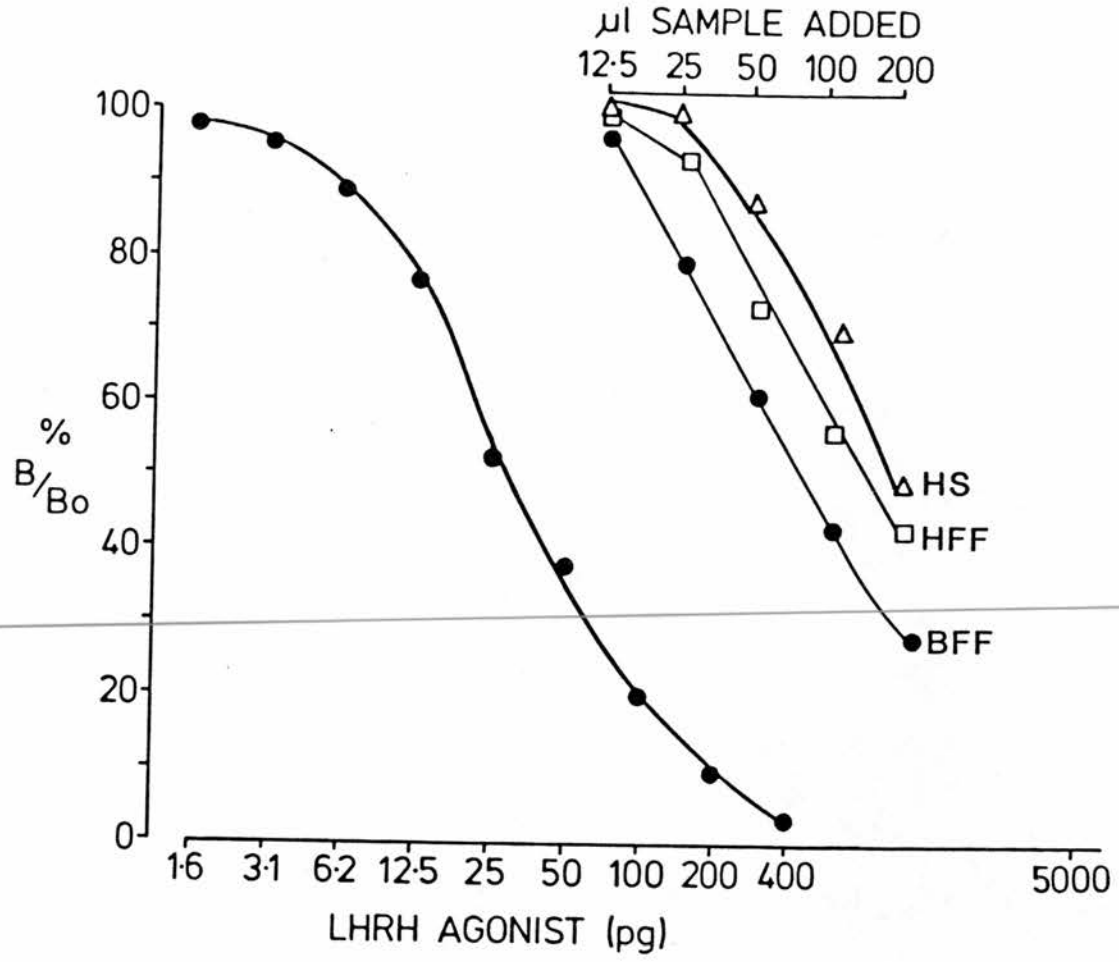

FIGURE 8.5 Cross reactivity of LHRH agonist (●—●) and acid-ethanol extracts of human follicular fluid (HFF), bovine follicular fluid (BFF) and human serum (HS) after Amicon ultrafiltration, in an RIA based on antiserum R103.

Each point represents mean of duplicate determinations.

each of 8 wells in the Amicon apparatus.

Samples of human and bovine follicular fluid, with human serum as a control, were extracted using acid-ethanol (see 8.4.1). The residue was taken up in distilled water and subjected to ultrafiltration. The osmolality of the eluate was determined at various time intervals using an osmometer and the samples removed only when a value similar to that obtained from distilled water alone was reached. In practice this took 6-9 hours. The contents of the wells were then lyophilized and reconstituted in assay buffer to a final concentration of 1 ml:6 mls original sample volume.

Results:- Despite pre-soaking in 5% BSA the Amicon filters were found to adsorb 40% of added ^{125}I-LHRH agonist.

Both human and bovine follicular fluid extracts displaced binding in all 3 assays (Figs. 8.3; 8.4; 8.5); serum extracts were negative in RO and Nett assays (Figs. 8.3; 8.4) but interfered in the R103 assay (Fig. 8.5).

Problems:- 1) Adsorption of ^{125}I-LHRH on filters.

2) Prolonged extraction time at room temperature.

3) Presence of LHRH-like and LHRH agonist-like material in follicular fluid extracts.

4) Serum interference in R103 assay.

8.4.4 Solid phase purification using Sep-pak columns

A relatively simple, rapid method for the extraction and purification of peptides had been described using the ability of octadecasilyl silica to adsorb peptides from large volumes of aqueous solution and then release them into solvent mixtures such as methanol (Bennett et al., 1977).

Two methods were adopted and both enabled extraction of 80% of ^{125}I-LHRH agonist from serum.

8.4.4.1 Method A

This method had been utilized successfully to extract peptide fragments from somatostatin and corticotropin from both fluid and tissue samples (Bennett et al., 1977; 1978). Rat ovarian tissue (from immature and PMSG-treated rats) and liver tissue were homogenized on ice (10 mls/g) in a solution of 5% formic acid, 15% trifluoroacetic acid (TFA as a deproteinizing agent) in IN hydrochloric acid (HCl). After centrifugation at 2500 rpm for 30 minutes at 4°C to pellet the proteins and tissue debris, 5 ml samples of supernatant were applied slowly (5 minutes loading time) to a Sep-pak 1 ml disposable column (Millipore Waters Assoc., Mass. U.S.A.). The column had been pre-washed with methanol and distilled water. The eluate was passed through the column a second time.

After sample loading the column was washed through with 5 mls 1% TFA, and the eluate, from the final wash with 5 mls of methanol:water:TFA (80:19:1 v/v) solution was collected and evaporated to dryness under nitrogen. The residue was taken up in assay buffer for RIA using antisera R0 and R103 to a final concentration equivalent to 200 mg wet weight of tissue per ml. A control solution of extracted distilled water was also assayed. This had been passed through the column as described.

Results:- None of the extracts cross-reacted in the R0 assay and only the data from R103 is shown in Fig. 8.6. Control fluid was negative but both ovarian and liver extracts cross-reacted with the antiserum.

Problem:- Liver 'control' tissue cross-reacted in R103 assay.

8.4.4.2 Method B

This method had been employed for the extraction of met-enkephalin from human plasma and cerebrospinal fluid (Clement-Jones et al., 1980). The same Sep-pak columns as described above were employed, except that a

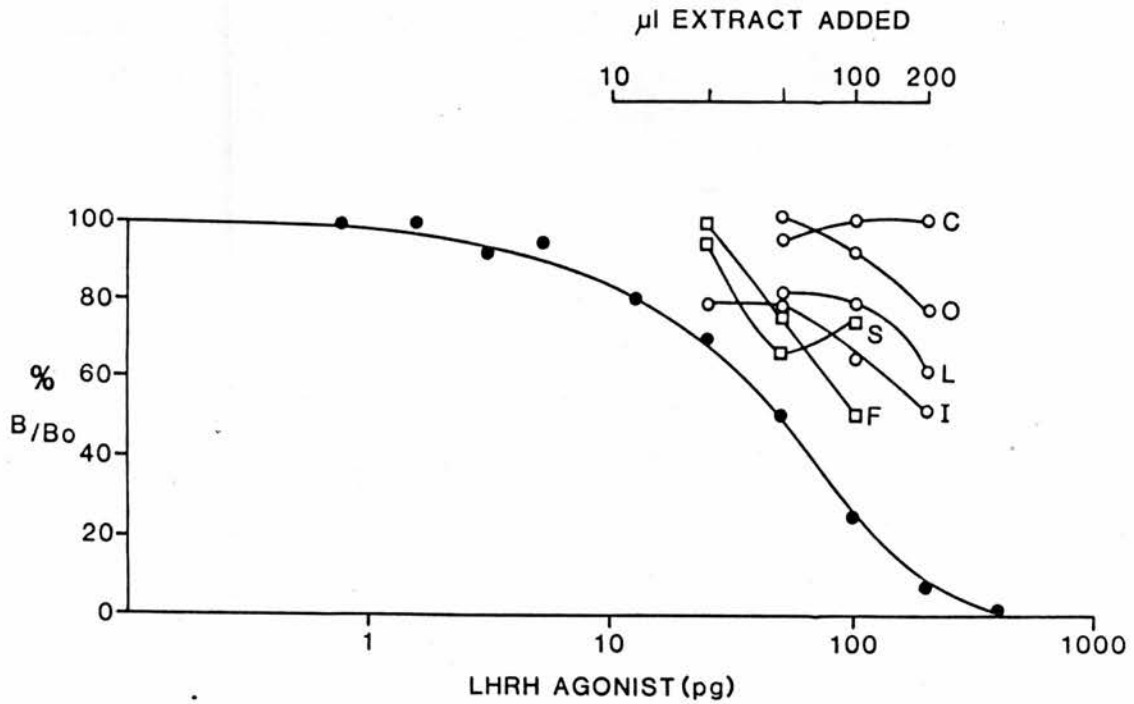

FIGURE 8.6 Cross reactivity of LHRH agonist (●—●) and Sep-pak extracted bovine follicular fluid (F) or serum (S) according to method 4.A (□—□) and Sep-pak extracted rat liver (L) control medium (C) and rat ovarian tissue 48 h after PMSG treatment (O) according to method 4.B (○—○), in an RIA based on antiserum R103.

Each point represents mean of duplicate determinations.

different elution procedure was utilized.

10 ml samples of bovine follicular fluid or serum were acidified with 0.1 ml formic acid and centrifuged at 2500 rpm for 30 minutes at 4°C. The supernatant was applied to a Sep-pak column. The column had been pre-washed in sequence with 1 ml 1% TFA, 1 ml methanol:TFA:water (80:1:19 v/v) solution, 1 ml 1% TFA and 1 ml 1% formic saline with 1.6% glycine in INHCl. After sample loading the column was washed with 1% formic acid followed by 1 ml 1% TFA and the sample eluted with 1 ml TFA:methanol:water solution. After evaporation to dryness the residue was taken up in assay buffer to a final concentration 6 times the original volume.

Results:- Neither of the extracts cross-reacted in RO assay. Both follicular fluid and serum extracts cross-reacted in the R103 radioimmunoassay (Fig. 8.6).

Problems:- 1) No cross-reactivity in RO assay (compare Fig. 8.3).

2) Serum interference in R103 assay.

8.4.5 Acetic Acid extraction

This method had been successfully utilized to demonstrate the presence of testicular LHRH-like material (Sharpe & Fraser, 1980b). Rat ovarian (from immature or PMSG primed rats) and liver tissues were homogenized in 10 mls of either 0.3 N or 0.02 N acetic acid immediately after removal from the animal and boiled for 15 minutes to inactivate any remaining functional enzymes. After centrifugation at 2500 rpm for 30 minutes at 4°C the supernatant was lyophilized and the residue taken up in assay buffer to a final concentration equivalent to 200 mg wet wt/ml.

Results:- Control extracts of boiled, lyophilized acetic acid were without activity in all assays (Figs. 8.7; 8.9). Both liver and ovarian extracts from 0.02 N acetic acid extraction cross-reacted in RO (Fig. 8.7)

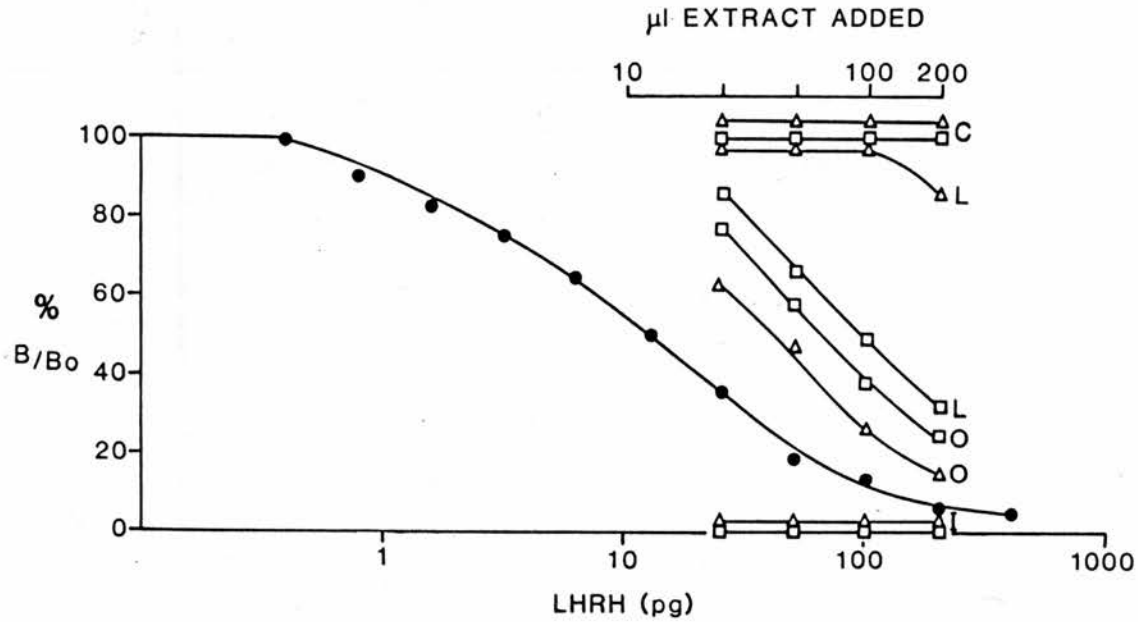

FIGURE 8.7 Cross reactivity of LHRH (●—●) and extracts of ovaries from immature rats (I) or from rats 48 h after PMSG treatment (O) and rat liver (L) prepared by extraction with 0.3 N acetic acid (△—△) or 0.02 N acetic acid (□—□) with acid controls (C), in an RIA based on antiserum RO.

Each point represents mean of duplicate determinations.

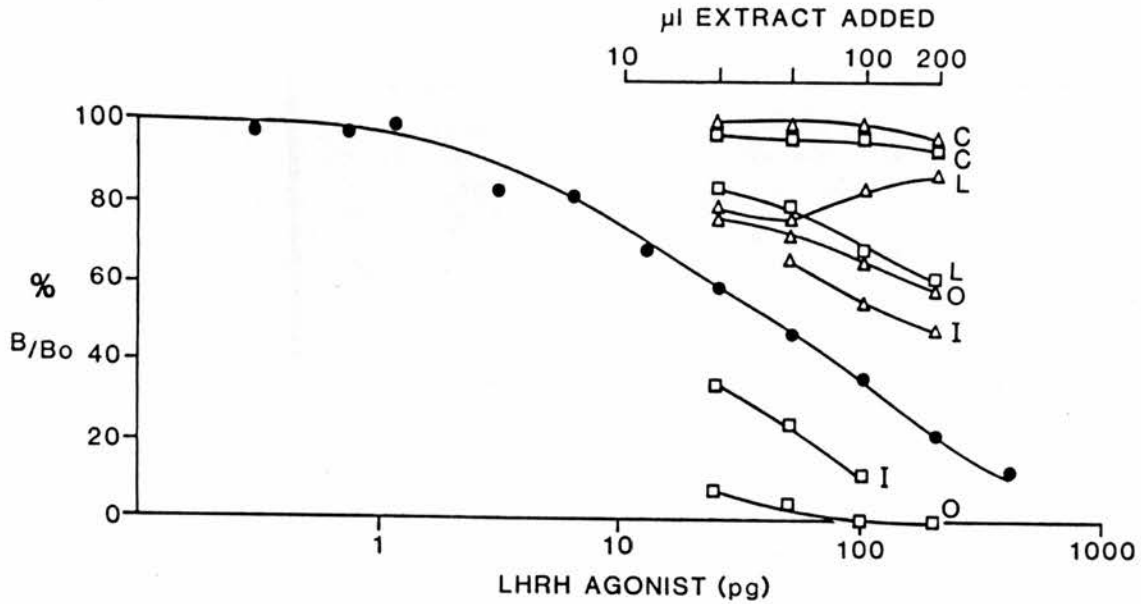

FIGURE 8.8 Cross reactivity of LHRH agonist (●—●) and extracts of ovaries from immature rats (I) or from rats 48 h after PMSG treatment (O) and rat liver (L) prepared by extraction with 0.3 N acetic acid (△—△) or 0.02 N acetic acid (□—□) with acid controls (C), in an RIA based on antiserum R103.

Each point represents mean of duplicate determinations.

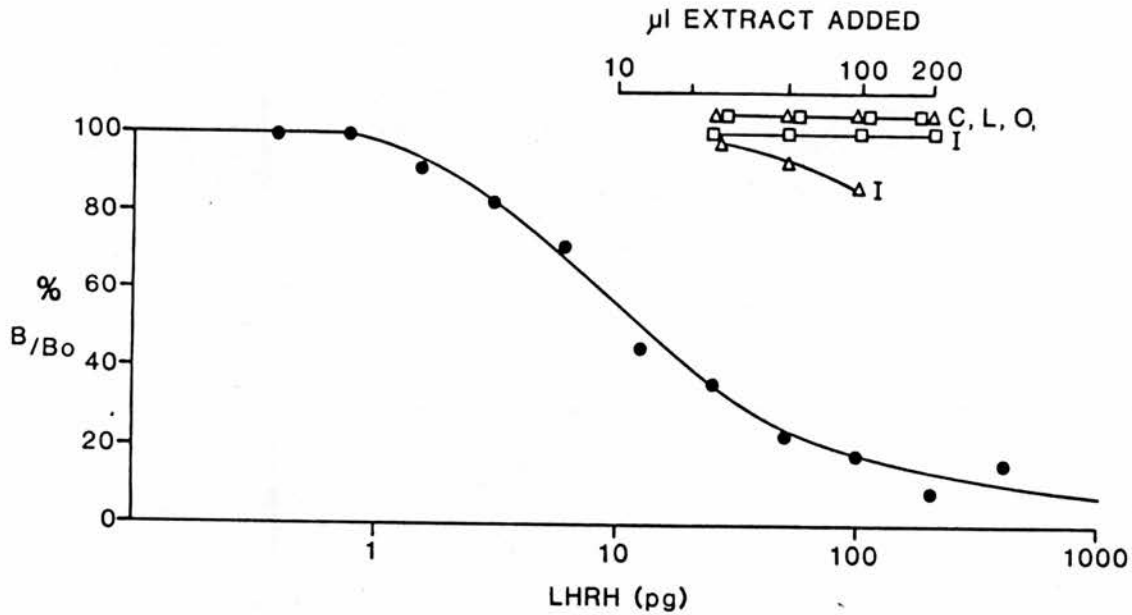

FIGURE 8.9 Cross reactivity of LHRH (●—●) and extracts of ovaries from immature rats (I) or from rats 48 h after PMSG treatment (O) and rat liver (L) prepared by extraction with 0.3 N acetic acid (Δ—Δ) or 0.02 N acetic acid (□—□) with acid controls (C), in an RIA based on the Nett antiserum.

Each point represents mean of duplicate determinations.

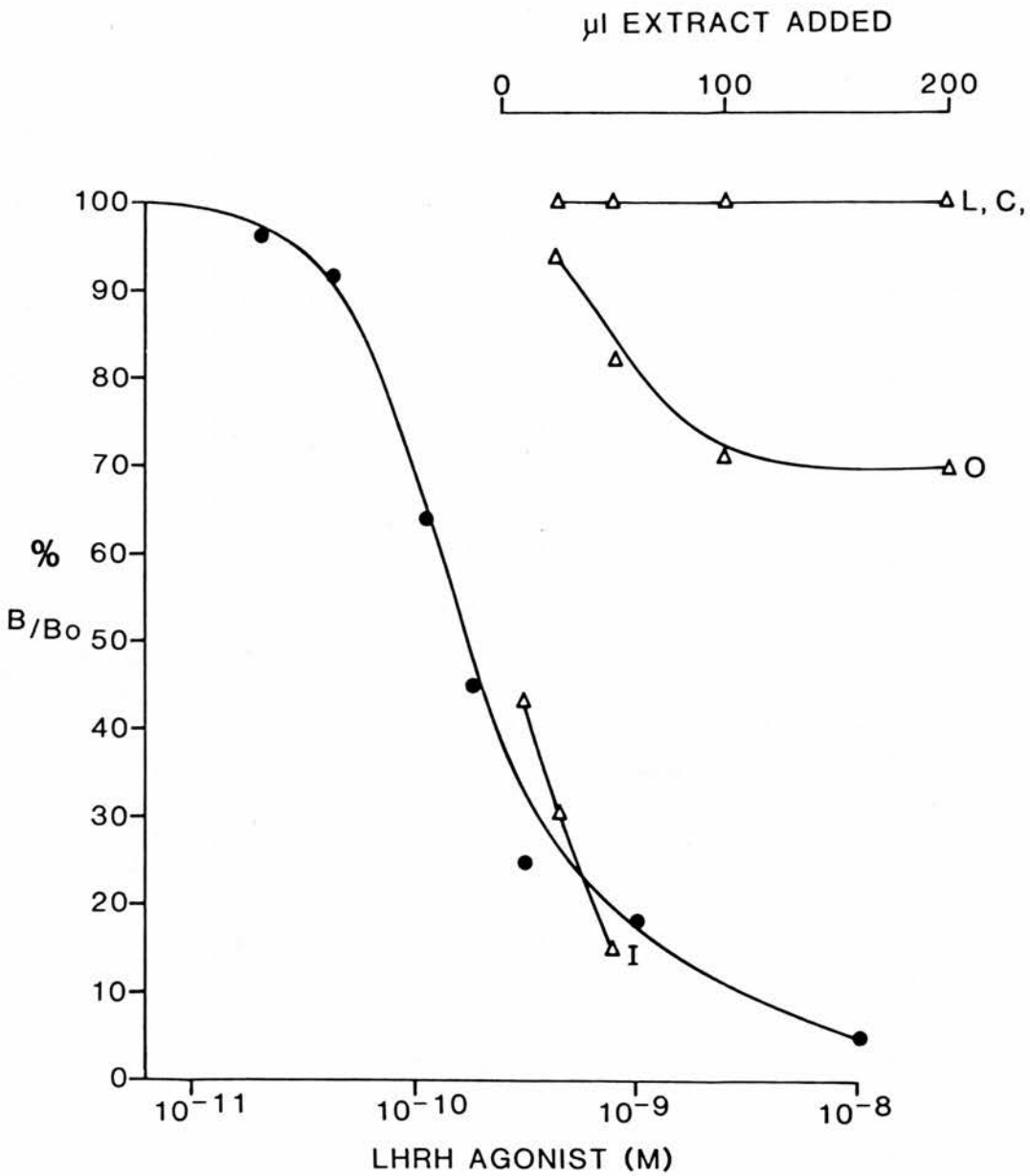

FIGURE 8.10 Displacement of ^{125}I-LHRH agonist binding to rat ovarian tissue by LHRH agonist (●—●) and 0.3 N acetic acid extracts of ovaries from immature rats (I) or from rats 48 h after PMSG treatment (O) and rat liver (L) with acid control (C).

Each point represents mean of duplicate determinations.

and R103 (Fig. 8.8) assays, but not in the Nett assay (Fig. 8.9).

Ovarian tissue extracted with 0.3 N acetic acid cross-reacted in RO (Fig. 8.7) and R103 (Fig. 8.8) assays, liver extracts gave minimal displacement. Only extracts of immature ovaries showed a minimal displacement in Nett assay (Fig. 8.9).

These extracts were also tested in the ovarian RRA (Fig. 8.10). Both liver and acid controls showed no displacement whereas both ovarian extracts displaced binding.

Problems:- 1) Non-parallel displacement of binding in assays.

2) Repeatability of results. Additional experiments using these procedures (i.e. 0.3 N acetic acid) gave either essentially the same result or no activity at all in any of the assays.

3) Immature ovarian tissue totally abolished binding in R103 and RO but not Nett or RRA.

8.5 DISCUSSION

The results presented in Figs. 1-10 are an illustration of some of the problems encountered whilst attempting to demonstrate the existence of a specific LHRH-like peptide in ovarian tissue. The most common problem was that of interference of 'control' extracts from liver or serum. Several methods were tried in an attempt to extract the peptide. Since concentrating may have increased non-specific interference such as salt, methods to remove salt were tried. The only techniques which resulted in minimal 'control' interference were, UM05 ultrafiltration and 0.3 N acetic acid extraction. The latter method appeared to be the most promising, extracts indicating the presence of LHRH-and LHRH agonist-like material (cross-reactivity in RO and R103 assays) non-identical to LHRH (no effect in Nett assay) in ovarian, but not liver tissue. Immature ovarian tissue

obliterated binding in R103 and RO assays which may indicate a high concentration of material in this tissue compared with PMSG primed tissue. However, another major problem encountered with 0.3 N acid extraction was that of repeatability. The same procedure was repeated numerous times, either revealing a similar pattern to that described or no activity at all in any extract. Thus no concrete data was obtained as to the presence or nature of a putative LHRH-like factor in the ovary.

Several points must be borne in mind when assessing data on LHRH-like factors:-

1) Ovarian LHRH receptor concentrations are 25% those of the pituitary (see Chapter 3). Therefore since it is likely that a putative endogenous ligand is present in very small quantities, extraction procedures consequently need to utilize large amounts of tissue. Use of the subsequent 'concentration' of crude extracted material inevitably risks inducing abnormally high levels of non-specific substances such as salt which may interfere with the assay systems used. It is therefore essential to include data from control extracts in order to assess the tissue specificity of the data presented. In the experiments reported, tissue was concentrated to a final level equivalent to 200 mg wet wt/ml and fluids concentrated 3-6 times. It could be suggested either that these levels were insufficient to provide enough peptide material or that the concentration factor was too great to exclude non-specific interference. Relatively few studies in the literature have included data on control extractions (Table 8.1).

2) If the putative intragonadal LHRH-like factor is a small locally produced and acting peptide it is likely to be degraded rapidly, thus

hampering extraction efficiency. This may explain the variability in the data obtained (Figs. 1-10). Degradation was minimized in these studies by performing all rat tissue extractions immediately after removal from the animal under enzyme-inactivating or protein-denaturing conditions. However, with the follicular fluids obtained from slaughter house or hospital sources there was considerable delay between removal of the follicular fluid from the female, and extraction. Degradation of peptides by endogenous peptidases had most likely occured prior to extraction of these samples.

3) The current available data suggests that extra-hypothalamic factors are LHRH-like. The majority of extraction procedures utilized have been based on the assumption that the molecule of interest is a small molecular weight peptide (see Table 8.1). However, this may not be the case. It is already clear from a number of studies that hypothalamic LHRH is present in a number of molecular forms, assumed to be precursor or prohormones, and is therefore a heterogeneous molecule (Barnea & Porter, 1975; Sternberger et al., 1981; Gautron et al., 1981). The same characteristic has been suggested for testicular LHRH. Dutlow & Millar (1981) have suggested the presence of multiple forms of LHRH-like activity from acid-extracts of the testis which, when subjected to affinity chromatography and Sephadex G100 separation gave 4 immunoreactive peaks with apparent molecular weights ranging from $< 4,000$ to $> 100,000$. Two forms of testicular LHRH with widely differing molecular weight were also suggested by Bhasin et al., 1983.

Thus, since the chemical identity of the putative endogenous ligand is uncertain, the extraction procedures employed in these experiments

may be inappropriate for the nature of the ligand.

4) Although it is of primary importance to rule out the possibility that the observed characteristics of extracted material are due to non-specific factors, it is uncertain what appropriate 'control' tissues to include. Serum has been indicated to contain immunoreactive LHRH (e.g. Miyake et al., 1980; Sarda et al., 1981). A macromolecule cross-reacting with LHRH antisera has been located in liver (Barnea & Porter, 1975) and direct effects of LHRH have been reported in kidney (Lecomte et al., 1982) and adipose tissue (Murthy & Modesto, 1974). LHRH receptors have been reported in the adrenal gland (Bernardo et al., 1978).

In addition, since LHRH may be a neurotransmitter in the sympathetic nervous system (see Chapter 1) detection of LHRH-like material in tissues may only reflect sympathetic innervation of that tissue.

8.6 CONCLUSIONS AND FUTURE PROSPECTS

From the data presented in this Chapter it is evident that many problems were encountered during the investigation of gonadal LHRH-like peptides and no firm conclusions could be made as to the existence of an intragonadal LHRH-like factor.

Other investigators have attempted to isolate ovarian LHRH and repeat the observations of Ying & Guillemin (1980) but have met with little success. Thus immunohistochemical localization of ovarian LHRH-like material has been unsuccessful (Moss et al., data reported in Smith-White & Ojeda, 1983) as have further radioimmunological methods (Ojeda, unpublished observations cited in Smith-White & Ojeda, 1983). However, LHRH bioactivity has been suggested to be present in dextran-charcoal treated peritoneal fluid but not serum (Demoulin et al., 1981). Table 8.1

TABLE 8.1

Summary of some of the data available in the literature on methods of extracting gonadal LHRH-like factors and their characteristics.

Tissue	Extraction/Purification	Assay	Comment	Reference
Ovary, follicular fluid	acid/EEA/dialysis/HPLC	bioassay RIA	data not repeatable ? due to contamination	Ying et al., 1981 Esch et al., 1983
Follicular fluid	acid/ethanol Amicon filtration	bioassay	no serum control	de Jong et al., 1979
Milk	acid/methanol, HPLC	bioassay RIA	no control fluid	Amarant et al., 1982
Testis & Liver	acid/ethanol, HPLC	RIA, RRA, bioassay	broad peak activity Liver extract interference after HPLC	Fraser et al., 1982 Sharpe & Harmer, 1983
Testis	acid, immunoaffinity chromatography Sephadex G100	RIA	Mr $>$100K, 32K, 5K, $\leq$ 4K no control tissue	Dutlow & Millar, 1981
Testis & Kidney	ethanol/CCl_4/acid immunoaffinity chromatography, HPLC	RRA, RIA	Mr 68K & 6K	Bhasin et al., 1983
Sertoli cell culture medium	acid/methanol, diethyl ether	RIA bioassay	no medium control	Nagendranath et al., 1983

provides a summary of some of the data presented in the literature on LHRH-like peptides, with a number of studies failing to include 'control' data.

Due to the pitfalls inherent in RIA and RRA methods, particularly interference by non-peptide molecules, perhaps a more promising approach would be to utilize an ovarian bioassay for ovarian extracts, namely isolated follicles (Chapter 7). The bioactivity as regards stimulation of basal androgen and progesterone could be assessed together with the ability of LHRH antagonists and/or peptidases to inhibit the effect.

The problems encountered in the experiments detailed earlier have been echoed by a recent report concerning the observations of Ying et al., 1981. The group has failed to repeat the demonstration of a "gonadocrinin" (Esch et al., 1983). They report "occasional evidence" for activity (see 8.4.5) and similar activity was found to be present in 'control' tissues, lung and kidney, even after purification by HPLC. (A similar problem has been encountered with testicular extracts following HPLC - see Sharpe & Harmer, 1983). In addition, activities similar to LHRH and LHRH agonist were reported (Esch et al., 1983; Sharpe & Harmer, 1983).

Esch et al., (1983) suggested several explanations for their data. Firstly that the rat ovary contains two molecules, LHRH and LHRH agonist (although reservations were expressed as to the biological equivalent of DTrp). Secondly, that activity in 'control' tissues represents insignificant production of minute amounts of the molecule due to occasional derepression of the gene. Finally that the data published previously on the isolation of gonadocrinin was an artefact due to contamination of the extracts with LHRH (Esch et al., 1983). They

concluded "it may thus be still true that gonadal tissues do contain LRF-like peptides. If this should eventually be proven beyond doubt, our early conclusions would deserve no scientific credit since we can not exclude the possibility that we are dealing with an artefact".

In conclusion, therefore, evidence for the existence of an intra-ovarian LHRH-like factor is not convincing. This chapter has attempted to indicate some of the pitfalls inherent in its isolation and assay. To date, no adequate characterization of such ovarian material has been achieved and in the majority of reports so far published, the contribution of non-specific interference has either not been studied, or cannot be excluded.

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9

GENERAL DISCUSSION

9.1 PITUITARY LHRH RECEPTORS

9.2 OVARIAN LHRH RECEPTORS

9.3 DIRECT OVARIAN ACTIONS OF LHRH AND LHRH AGONIST

9.1. PITUITARY LHRH RECEPTORS

The first part of this thesis was concerned with a consideration of the mechanisms governing changes in LHRH receptors. Pituitary LHRH receptor concentrations were markedly altered by experimental regimes associated with an interference with endogenous LHRH release, such as immunoneutralization of LHRH. It was therefore suggested that receptor autoregulation was an important means of controlling pituitary LHRH receptors (Chapter 4). However, the relevance of these observations as regards the antifertility effects of LHRH and LHRH agonist or the control of pituitary responsiveness under physiological conditions is unclear.

One of the mechanisms suggested to explain the antifertility effects of LHRH agonist was a decreased pituitary responsiveness due to decreased or down-regulation of LHRH receptors (Chapter 1). Chronic administration of a range of doses of LHRH agonist resulted in an impairment of reproductive function despite the fact that pituitary LHRH receptors were within the cyclic range (Chapter 4). It seemed unlikely therefore that receptor down-regulation could explain the noted antifertility effects. This conclusion is supported by examination of the doses of LHRH agonist required to elicit receptor down-regulation compared with those causing antifertility effects. Decreased pituitary receptor concentrations (a net loss of only 32%) occurred only after 6 day infusion with 30 ng LHRH agonist (Clayton, 1982) and thus the 'threshold' for receptor down-regulation appears to be extremely high. In contrast, much lower doses of LHRH agonist 0.2 ng, Arimura *et al.*, 1980 and 1 ng (Johnson *et al.*, 1976a; Vilchez-Martinez *et al.*, 1979) per day have been reported to

inhibit reproductive function. In conclusion, antifertility effects of LHRH agonist are not invariably correlated with decreased pituitary LHRH receptors. However, pituitary responsiveness is clearly impaired following prolonged LHRH administration, as was shown by the failure of oestrogen to induce an LH surge in treated ewes (e.g. Rippel et al., 1974). In addition, the ability of the pituitary to respond to exogenous oestrogen-induced positive feedback was abolished, delayed or reduced in macaque monkeys treated chronically with LHRH agonist (Fraser, 1981b). It is likely therefore that decreased pituitary responsiveness can occur by mechanisms other than LHRH receptor down-regulation, e.g. post-receptor mechanisms involved with synthesis and secretion of gonadotrophins.

The relevance of receptor down-regulation in the control of pituitary LHRH receptors under physiological conditions is also questionable. Removal of endogenous LHRH did not result in an increased receptor concentration indicative of removal of a negative regulator. In addition, abolition of the pro-oestrous surge of LHRH by immunoneutralization, failed to prevent the receptor decrease at oestrus (Chapter 4). It is likely therefore that reduced LHRH receptor numbers under physiological conditions are due, either to the influence of heterologous ligands such as steroids (as implicated by Giguere et al., 1981; Adams et al., 1981a) or to removal of LHRH as a positive regulator of its own receptors. Endogenous LHRH concentrations are unlikely to reach the high threshold values necessary for receptor down-regulation, under physiological conditions.

The majority of evidence presented in this thesis and in the

literature suggests that LHRH autoregulation is primarily one of up-regulation i.e. a maintenance or increase in receptor numbers. Thus administration of more physiologically representative doses of LHRH or LHRH agonist resulted in increases in pituitary receptor numbers both *in vivo* (e.g. Frager *et al.*, 1980; Dalkin *et al.*, 1981; Naess *et al.*, 1981; Heber *et al.*, 1982; Marchetti *et al.*, 1982; Clayton, 1982; Pieper *et al.*, 1982) and *in vitro* (Loumaye & Catt, 1982; Smith *et al.*, 1983). In addition, receptor changes appeared to mirror those of known or assumed changes in endogenous LHRH levels; thus when pituitary exposure to endogenous LHRH was reduced (by active or passive immunization against LHRH), LHRH receptors were reduced, and conversely when LHRH output was assumed to be elevated, e.g. after castration receptor numbers were increased (Chapter 4).

Whereas the mechanism by which receptor down-regulation can occur is largely acknowledged to be via receptor mediated endocytosis and degradation of the hormone-receptor complex (see Goldstein, 1979), the mechanisms for receptor up-regulation are unknown. Since LHRH receptors are known to be internalized after exposure to LHRH (see Chapter 3) the fact that receptor numbers are unchanged or even increased following LHRH administration, implicates a rapid receptor turnover. Application of cycloheximide with LHRH, resulted in a decreased receptor levels below that of controls, indicating continual synthesis and turnover of LHRH receptors under normal conditions (Smith *et al.*, 1983).

Several mechanisms can be postulated to account for increased receptor numbers following ligand exposure. These are summarized

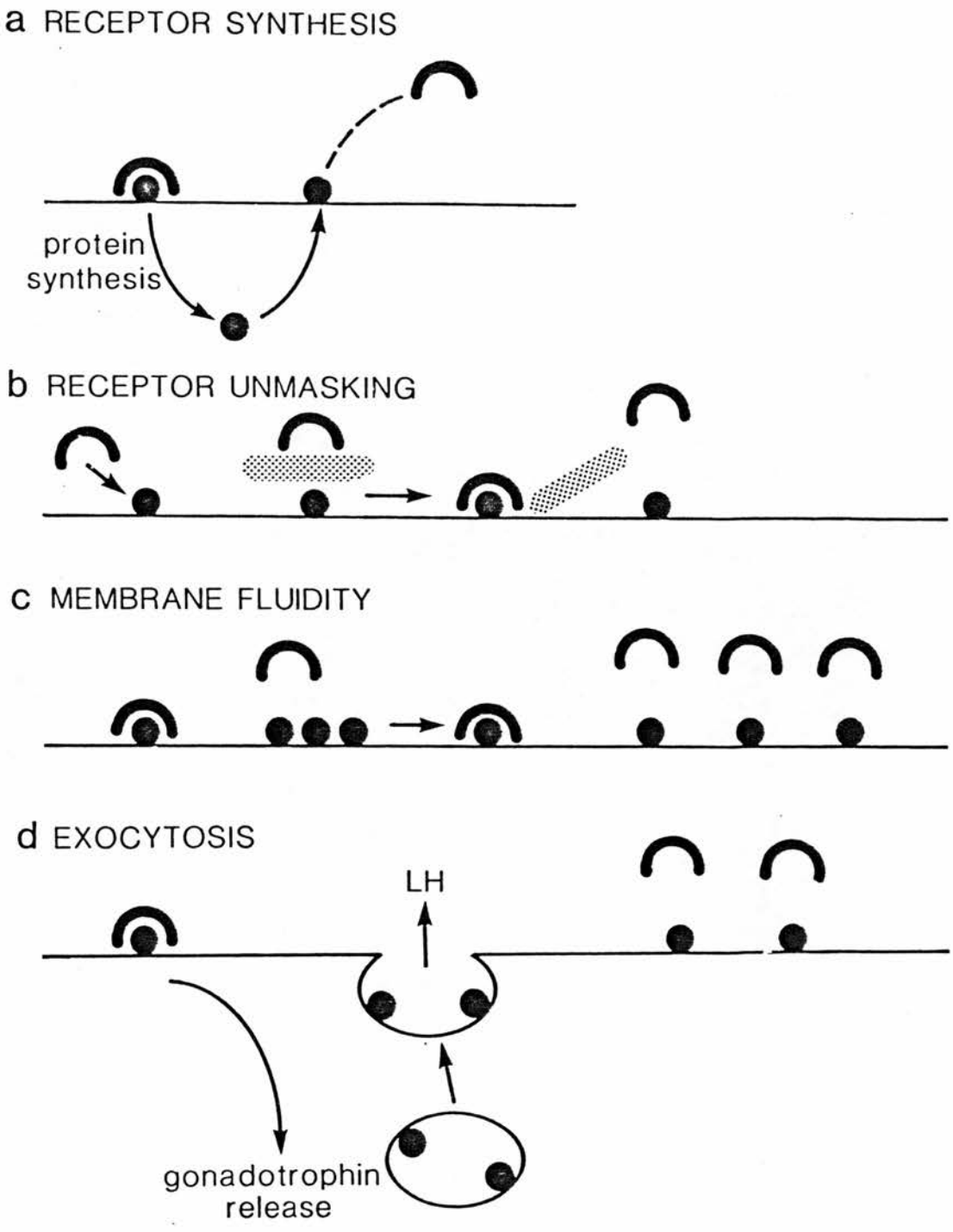

FIGURE 9.1 Diagramatic representation of suggested mechanisms by which interaction of LHRH ( ) with its pituitary receptor (●) could cause increases in the concentration of receptors seen at the cell surface. For details see text.

diagramatically in Fig. 9.1. The first mechanism involves LHRH stimulation of cellular protein synthesis (a phenomenon noted by Hsueh & Jones, 1982b) and production of its own receptors (Fig. 9.1a). Since cycloheximide abolishes receptor up-regulation *in vitro* (Smith *et al.*, 1983) this mechanism is clearly a valid one. It would be interesting to determine whether or not the dioestrous to pro-oestrous receptor rise is due to de novo synthesis of receptor protein.

Secondly, a more rapid response could be envisaged if interaction of LHRH with its receptor resulted in unmasking of other membrane receptors, either due to removal of an impeding molecule (Fig. 9.1b) or to changes in membrane fluidity mobilizing and exposing receptors (Fig. 9.1c). Finally, since LH receptors have been suggested to be present on membranes of exocytotic secretory granules (Suter *et al.*, 1980) and since pituitary uptake of LHRH occurred as long as LH release continued (Duello & Nett, 1980) it could be suggested that LHRH receptors are present on the membranes of LH-secretory granules, being incorporated into the plasma membrane as LH is secreted (Fig. 9.1d). In agreement with this hypothesis, LHRH receptors have been located on the limiting membrane of secretory granules (Sternberger & Petrali, 1975).

It is important to realise however that although LHRH receptor up-regulation occurs, changes in receptor numbers are not invariably correlated precisely with endogenous LHRH levels. For example, during the oestrous cycle, receptor numbers increase from the afternoon of dioestrus one, well before the endogenous surge of LHRH in portal blood (see Fig. 9.2). Since immunoneutralization of LHRH at this time prevented the receptor rise (Chapter 4) it could be suggested that LHRH up-regulation

involves the qualitative pattern of LHRH release as well as the quantitative amounts released. This may also be the reason why the post-castration receptor increase (see Chapter 4) precedes that of the reported increase in endogenous LHRH (e.g. Sarkar & Fink, 1980).

It is a generally accepted principle that where tissue receptors are present in excess, changes in receptor numbers will alter, not the maximum potential response, but the responsiveness of that tissue to the ligand (e.g. Catt et al., 1979 for review). Initial studies confirmed a correlation between pituitary LHRH receptors and pituitary responsiveness to LHRH (reviewed in Chapter 4). However, a number of more detailed studies have implied that the relationship is not a simple one. Thus as discussed earlier the decrease in pituitary responsiveness following chronic LHRH agonist administration, was not correlated with decreased receptors. In addition, studies in the male rat, described in Chapter 4, showed no close correlation between pituitary LHRH receptors and serum LH, following passive immunization against LHRH. Receptor recovery preceded that of serum LH and testosterone.

Similar observations can be made from a more detailed look at the correlation between receptor concentrations and pituitary responsiveness to LHRH during the rat oestrous cycle. Fig. 9.2 details data (centred round the time of the LH surge) on receptor numbers (from Clayton et al., 1980), endogenous LHRH release (from Levine & Ramirez, 1982) and pituitary responsiveness to a set dose (50 ng/100 g wt) of exogenously administered LHRH (from Aiyer et al., 1974a). Receptor numbers are maximal at the time of maximal responsiveness (namely pro-oestrus) and minimal at the time of least responsiveness (i.e. oestrus). However,

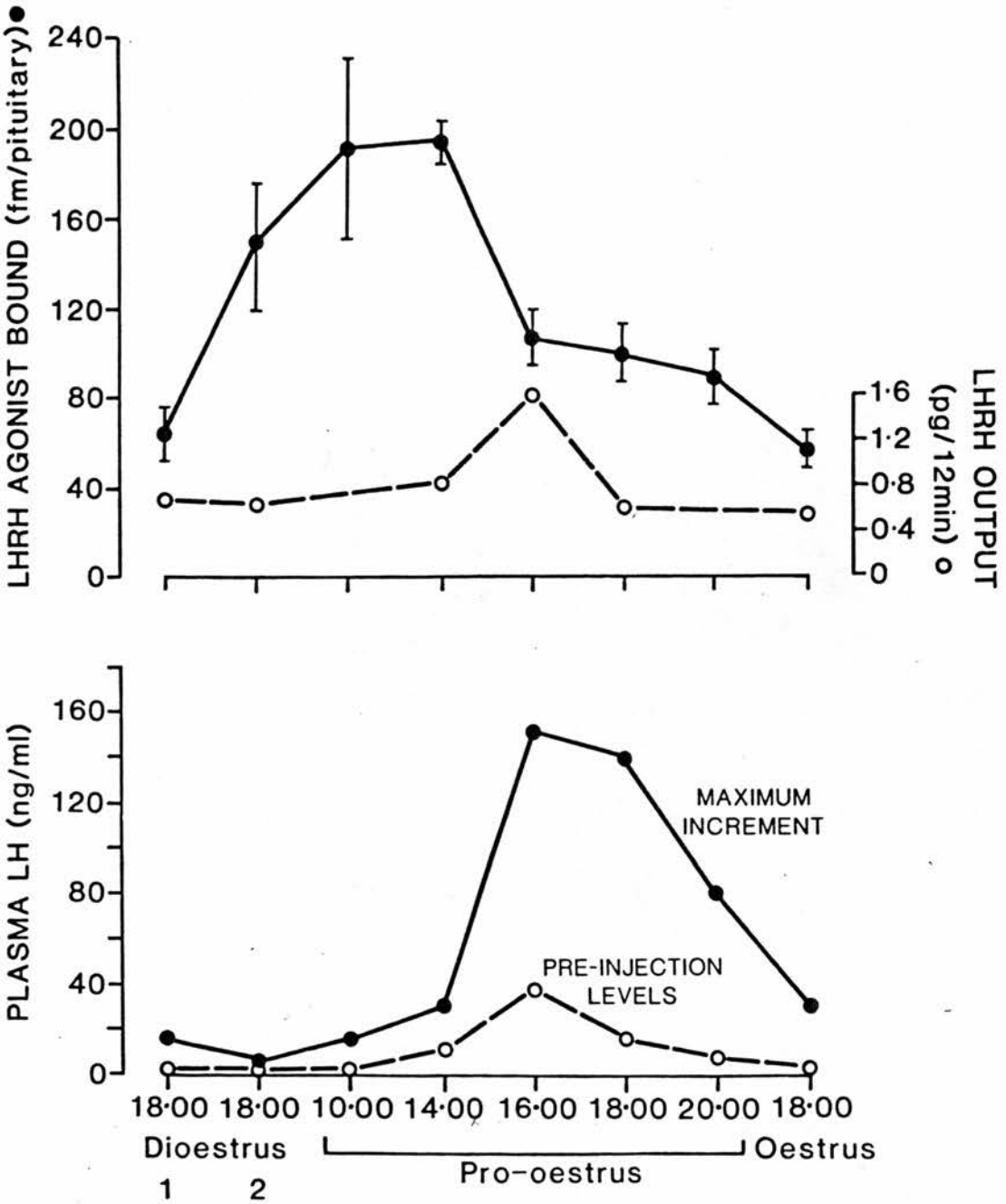

FIGURE 9.2 Summary of data obtained from a number of sources on changes during the rat oestrus cycle in pituitary LHRH receptors (from Clayton et al., 1980), endogenous LHRH output (from Levine & Ramirez, 1982) and pituitary responsiveness to a 50 ng dose of LHRH as regards LH release (Aiyer et al., 1974a).

receptor numbers are increased to pro-oestrous levels on dioestrus day 2, yet pituitary responsiveness is low. This data also indicates that pituitary responsiveness is not invariably correlated with changes in LHRH receptors and implies that other factors are involved.

This conclusion has been reached by a number of others. Pituitary cells isolated from rats from different stages of the oestrous cycle showed the expected differences in responsiveness to LHRH. However, similar differences in the amount of LH released in response to a different secretagogue, namely potassium (K^+), were found. This data also indicated that pituitary responsiveness, as regards LH release, is independent of LHRH receptor changes (Speight & Fink, 1981).

Pituitary desensitization occurred after continuous exposure to LHRH in vitro (Smith & Vale, 1981; Badger et al., 1983). This was not due to depleted LH stores since further LH release could be induced by K^+ (Smith & Vale, 1981). It was expected therefore that this phenomenon was due to loss of LHRH receptors similar to the loss of epidermal growth factor (EGF) receptors noted after continuous exposure of cells to EGF (Heldin et al., 1979; see Catt et al., 1979). A number of studies have attempted to correlate the two parameters using isolated pituitary cells for simultaneous measurement of receptor concentrations and LH release.

Initial studies showed a coincidence of pituitary desensitization with loss of LHRH receptors after continuous exposure of isolated cells to LHRH (Zilberstein et al., 1983). However, this was by no means a general finding. Thus receptor numbers decreased during culture, whereas responsiveness increased (Naor et al., 1980). Pituitary desensitization

induced by continuous exposure to LHRH, was accompanied by an increase in receptors (Smith & Vale, 1983). This latter observation, namely low responsiveness in the absence of receptor decrease, has also been shown in vivo (Heber & Swerdloff, 1980) and is analogous to the situation described on dioestrus two. In addition, the converse has been shown since the self-priming effect of LHRH was reported to be accompanied by a decrease rather than an increase in LHRH receptors (Ferland et al., 1981).

It is likely therefore that post-receptor events are important in determining pituitary gonadotrophin release (Heber & Swerdloff, 1980; Speight & Fink, 1981; Smith & Vale, 1983). These phenomena have been described for other systems e.g. insulin receptors (Heaton & Gelehrter, 1981) and alpha adrenergic receptors (Takeyasu et al., 1981).

However, these observations detailing a lack of correlation between receptor numbers and responsiveness, are based on two assumptions, namely that the receptor assay used determines the total number of available receptors and that these receptors are all functional. These criteria may not be valid in all cases. Thus, measurement of receptor numbers in homogenates may include internal receptors and give different values from those estimated from whole cells. However this is unlikely, since similar receptor changes have been observed using either isolated cells or homogenates during the oestrous cycle (see Clayton et al., 1980; Meidan & Koch, 1981). Alternatively decreases in receptor numbers may be apparent, (rather than representing true receptor loss), due to receptor occupancy by endogenous or added ligand (e.g. Smith-White & Ojeda, 1983). Finally it is possible that receptors are present in several forms within the cell membrane. It could be envisaged that receptors capable of binding LHRH

(and thus giving an apparent high receptor concentration value) are not functionally linked to the gondotrophin-releasing apparatus. In addition, receptors may be non-randomly distributed over the cell surface. Responsiveness could therefore be controlled by regulating the distribution and relative proportions of coupled and uncoupled receptors without altering total receptor concentrations. These possibilities might explain why receptor down-regulation is not invariably associated with periods of decreased responsiveness and why, likewise an increase in receptors does not necessarily confer increased responsiveness. Thus the receptor assay is unlikely to distinguish between functionally active receptors, or between changes in receptor microaggregation or coupling to calcium mobilizing proteins.

Moreover, it must be remembered that 'pre-receptor' regulatory mechanisms may be equally important in determining pituitary responsiveness to LHRH in vivo. A synthetic peptidase substrate was suggested to compete for binding to LHRH receptors (Kuhl & Baumann, 1981), an observation which may indicate the presence of plasma membrane-associated degrading enzymes regulating the effective concentrations of LHRH (whether of endogenous or exogenous origins). In this regard it is important to remember that the ^{125}I-LHRH agonist receptor assay only detects changes in numbers of high affinity LHRH receptors. The low affinity receptors, present in greater concentrations (see Chapter 3) may represent enzymes degrading LHRH, therefore changes in the concentrations of these receptors may also regulate pituitary responsiveness to LHRH in vivo.

In conclusion, although LHRH is clearly important for the maintenance

and 'up-regulation' of its pituitary receptors, the relative contributions of changes in receptor concentrations, together with pre- and post-receptor mechanisms in the regulation of pituitary responsiveness to LHRH in vivo, remain to be established.

9.2 OVARIAN LHRH RECEPTORS

Despite extensive investigations on the biochemical nature and characteristics of ovarian LHRH receptors (see Chapter 3) surprisingly little is known either about their physiological significance in terms of ovarian function, or their regulation. It was evident from data presented in Chapter 5 that ovarian LHRH receptors were not controlled in parallel with those of the pituitary. This is not particularly surprising in view of the widely different functions of both receptors.

The distribution of ovarian LHRH receptors revealed one of the underlying difficulties in assessing receptor changes - namely their distribution between all ovarian compartments (see Chapter 3). The limited data available on changes in receptor concentrations in individual cell compartments suggested that granulosa cell receptors were altered during puberty (Smith-White & Ojeda, 1983) and were regulated by both LHRH and FSH (Ranta et al., 1982). In addition, since active immunization against LH or LHRH resulted in increased receptor concentrations (Chapter 3) a role for LH in the long-term regulation of ovarian receptor concentrations was implicated. As yet however, no data is available on relative changes of receptor concentrations between individual ovarian compartments.

In addition, no studies have attempted to assess changes in the sensitivity of ovarian cells to LHRH. It is interesting to note that one

of the periods of highest receptor concentration (namely, during immaturity see Smith-White & Ojeda, 1981) was also a period when no short-term stimulatory effects could be determined in isolated follicles (Chapter 7), thus implicating either a role for post-receptor regulation or the presence of non-functional receptors at this time.

9.3 DIRECT OVARIAN ACTIONS OF LHRH AGONIST

Although direct effects of LHRH have been demonstrated on both follicular and luteal tissue (see Chapters 1 & 7) these studies have almost entirely utilized model systems to induce formation of ovarian tissue. Only one study has provided evidence for direct ovarian effects of LHRH in cycling rats, namely that of Corbin & Bex, (1981) showing induction of ovulation in hypophysectomized pro-oestrous rats. It would be of considerable interest to determine the steroidogenic responsiveness of rat follicles isolated during different stages of the oestrous cycle. Limited data reported in Chapter 7 indicated that follicles isolated from untreated immature rats were not responsive to LHRH agonist whereas those of a "dioestrous" of "pro-oestrous" type were. Ovarian responsiveness to LHRH may therefore vary during the life of the rat. In addition a recent report from Hillensjö et al., (1983) suggested that LHRH stimulated oocyte maturation in oocytes of small antral follicles but not pre-antral follicles.

Data presented in Chapter 7 emphasised a stimulatory role of LHRH and LHRH agonist with regard to both thecal and granulosa cell function (summarized in Fig. 9.3). These stimulatory effects were on basal rather than gonadotrophin induced events and contrasted to the predominantly

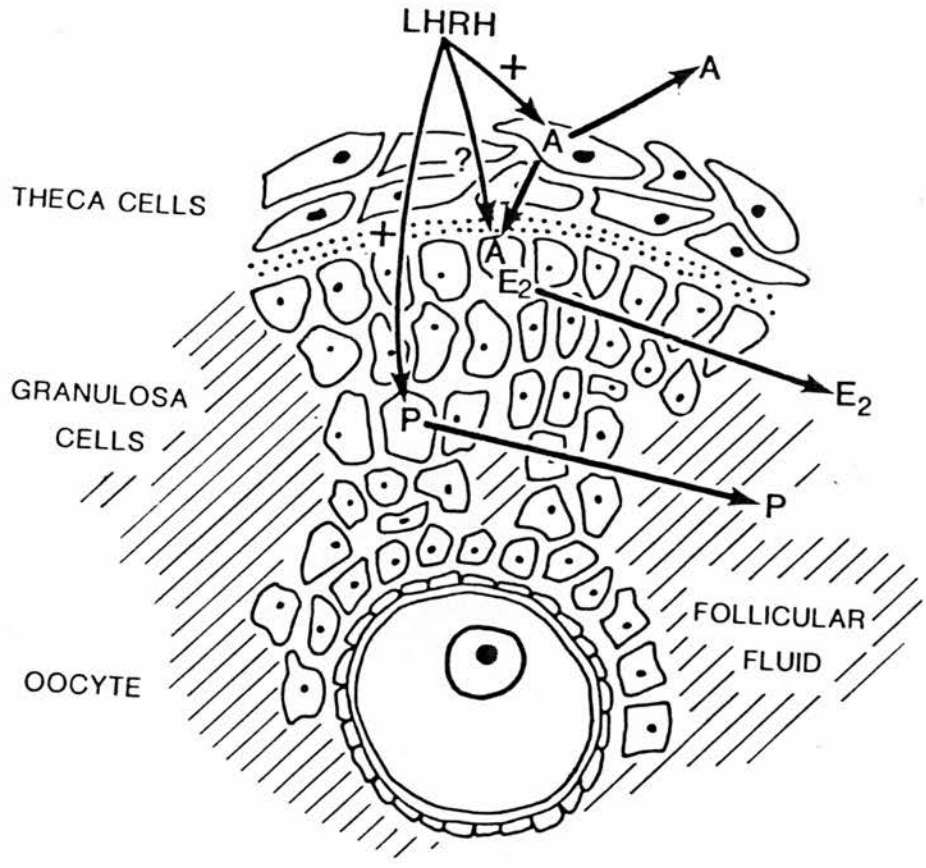

FIGURE 9.3 Summary of some of the stimulatory actions of LHRH on ovarian follicular function involving stimulation of basal androgen (A) progesterone (P) and possibly oestradiol (E_2) production.

inhibitory action seen in previous studies (see Chapter 1 and Fig. 1.1). Several differences are evident between the earlier studies indicating an inhibitory action of LHRH, and the data presented on stimulatory effects. Firstly, inhibitory effects were noted after prolonged incubation ($>$ 24 h) and were on gonadotrophin-induced events, basal changes not being reported (Table 9.1). In contrast, stimulatory effects were noted in short-term incubations ($<$ 20 h). Secondly, demonstration of inhibitory actions of LHRH on rat granulosa cell function have been confined exclusively to the model system of the immature rat (usually hypophysectomized) treated with diethyl stilbestrol (DES) (see Hsueh & Jones, 1981 for review). This model is known to induce pre-antral follicle development yielding large numbers of relatively undifferentiated granulosa cells, with minimal gonadotrophin stimulation (Hillier et al., 1977), by a direct effect of DES on the ovary, together with augmentation of subsequent ovarian responses to gonadotrophins (Meyer & Bradbury, 1960). In contrast, demonstration of stimulatory effects of LHRH or LHRH agonist have been confined exlusively to follicles or granulosa cells after follicular development had been induced by PMSG (Table 9.1). It could be suggested therefore that the responsiveness of follicular tissue to LHRH depends on prior exposure to gonadotrophins. Prior to and after initial exposure to gonadotrophins (in growing pre-antral follicles) LHRH exerts inhibitory actions, whereas after adequate gonadotrophin priming (e.g. in pre-ovulatory follicles) LHRH actions are stimulatory (see Fig. 9.4). This hypothesis has been confirmed by very recent studies by Naor et al., (1983) in which the effect of LHRH agonist on ovarian growth and

TABLE 9.1

Summary of direct effects of LHRH or LHRH agonist on follicular tissue

Gonadotrophin induced event	Basal event	Effect of LHRH/ LHRH agonist	Source of Tissue	Incubation Time	Reference
Progesterone		Inhibition	granulosa cells from hypophysectomized DES treated immature rat	48 h	Hsueh & Erickson, 1979a Knecht et al., 1981
Oestradiol		Inhibition		48 h	Hsueh et al., 1980
LH receptor		Inhibition		48 h	Hsueh et al., 1980
PRL receptor		Inhibition		48 h	Navickis et al., 1982
3 β HSD activity		Inhibition		48 h	Jones & Hsueh, 1982
Androsterone	Androsterone	Inhibition	cells isolated from dispersed immature rat ovary	4 days	Magoffin et al., 1981
	Progesterone	Stimulation	granulosa cells from PMSG primed rat	0-5 h	Clark et al., 1980
	Prostaglandins	Stimulation		5 h	Clark, 1982
	Progesterone	Stimulation	follicles isolated from PMSG primed rat ovaries	6-20 h	Hillensjö, 1981 & see Chapter 7
	Prostaglandins	Stimulation		6-20 h	Hillensjö et al., 1982
	Androstenedione/ Testosterone	Stimulation		3-8 h	see Chapter 7

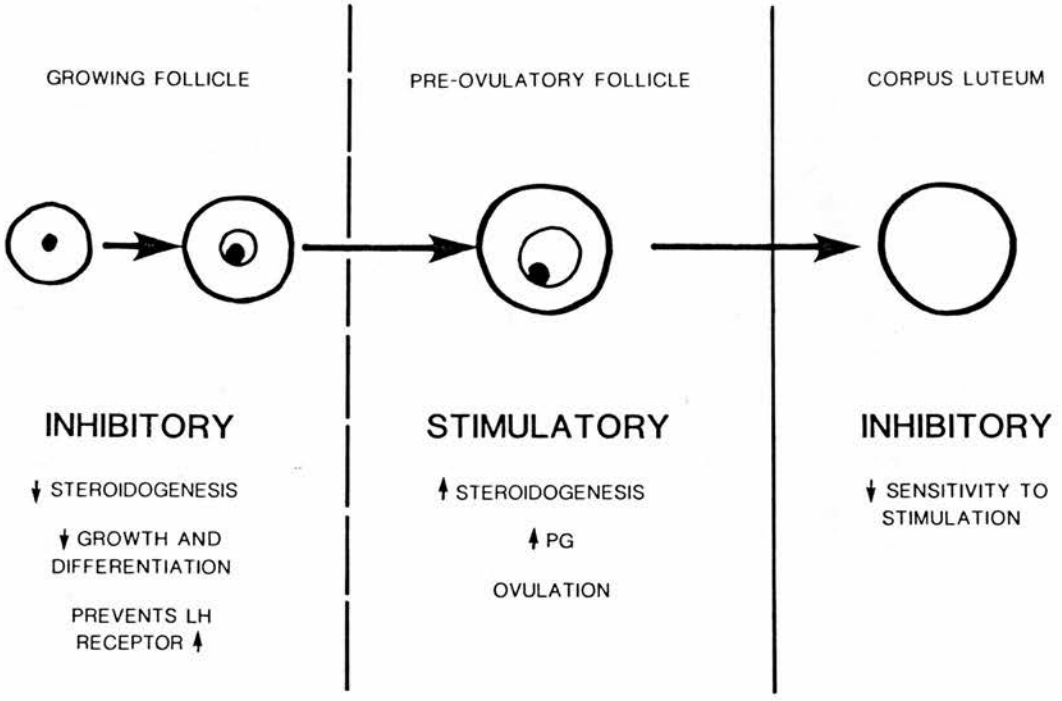

FIGURE 9.4 Schematic diagram indicating a possible role for LHRH as an intra-ovarian regulatory factor, derived from studies on responses to LHRH and LHRH agonist. Diagram from Fraser et al., 1983b.

ovulation was found to depend on the follicular stage of development rather than the dose or duration of exposure to LHRH. A non-gonadotrophin primed follicle was inhibited by LHRH agonist whilst a PMSG-primed follicle was stimulated (Naor et al., 1983).

Luteal tissue has been studied either as "granulosa-luteal" cells prepared by sequential treatment of granulosa cells (from hypophysectomized DES treated immature rats) with FSH and hCG (Jones et al., 1980) or from luteal tissue induced by PMSG/hCG treatment (Clayton et al., 1979a; Harwood et al., 1980; Behrman et al., 1980; Hall et al., 1981). LHRH has been found to be inhibitory to both basal- and hCG/LH-stimulated events using either model. It appears therefore that a fundamental difference exists between the responsiveness of luteal and follicular tissue. Prior to ovulation, LHRH appears to induce either stimulation or inhibition, after ovulation LHRH actions are entirely inhibitory (Fig. 9.4).

However, it must be emphasized that almost all the studies on direct ovarian actions of LHRH have utilized model systems in which specific ovarian states have been artificially induced. The physiological significance of these observations on the control of follicular growth, atresia and luteal function in vivo during the events of a normal oestrous cycle remain speculative.

The relevance of direct gonadal actions of LHRH to species other than the rat is unclear. Isolated mouse Leydig cells failed to respond to LHRH (Hunter et al., 1982). However, FSH-induced increases in progesterone and cAMP from porcine granulosa cells were inhibited by LHRH agonist (Massicotte et al., 1980). LHRH agonist stimulated progesterone

production from perfused rabbit ovaries in vitro (Koos et al., 1982) and inhibited LH-stimulated progesterone synthesis in isolated bovine luteal cells (Milvae & Hansel, 1980). Whether such direct effects occur in primates is unclear. Although no binding or direct effects have been reported in monkey corpora lutea (Asch et al., 1980; 1981) a recent report by Knecht cited unpublished evidence for a direct inhibitory effect of LHRH on progesterone production in cultured granulosa cells from FSH-treated monkeys (Knecht et al., 1983).

Data presented in Chapter 6 indicated the presence of specific binding sites for LHRH in human luteal tissue. These sites may also be present on human granulosa cells and mediate the LHRH agonist inhibition of progesterone production (Turek et al., 1982). Although the affinity of these human luteal binding sites was relatively low, and therefore their physiological significance uncertain, they may be activated by high levels of exogenously administered LHRH and LHRH agonist used clinically.

Finally, the question whether the gonad contains LHRH-like material was assessed in Chapter 8. No satisfactory data were obtained, and the question as to the existence of a specific intra-ovarian LHRH-like peptide remains unsolved. A new approach is required to investigate this phenomenon in view of problems encountered in the procedures described in Chapter 8. The most promising direction for the future lies with techniques of molecular biology. With the isolation of mRNA for rat hypothalamic LHRH (Curtis & Fink, 1983) it would be possible to produce a complementary, cDNA copy to utilize as a specific probe for the detection of such mRNA in a wide variety of tissues. Only when the existence, source and pattern of release of the postulated intra-ovarian LHRH-like molecule is known can the physiological significance of the direct ovarian effects presented in this thesis, be resolved.

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